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Thesis

How to cite:

Zulfiqar, Adnan (2016). The Development and Evaluation of Methods for the Screening of Steroids using Molecular Imprinted Polymers coupled with comprehensive Gas Chromatography/Mass Spectrometry and Aptamers coupled with Liquid Chromatography/Mass Spectrometry. PhD thesis The Open University.

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Version: Version of Record

Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.21954/ou.ro.0000bb5a>

oro.open.ac.uk

The development and evaluation of methods for the
screening of steroids using molecular imprinted
polymers coupled with comprehensive gas
chromatography/mass spectrometry and aptamers
coupled with liquid chromatography/mass spectrometry

A thesis submitted for the degree of

Doctor of Philosophy

by

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B.Sc. (Hons), Kingston University, 2003

31 August 2014

Department of Physical Sciences

The Open University

Declaration

I hereby certify that the work described in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously for a degree at this, or any other university.

Adnan Zulfiqar

Abstract

The profiling of hormones is an important requirement in sports dope testing and it is increasingly being used for clinical applications. The screening of samples for the detection of steroids is usually undertaken in urine and these analyses present many challenges. Issues related to the complexity of the matrix, the separation of exogenous from endogenous species and the very low detection limits have placed increasing demands on clinical and International Olympic Commission accredited laboratories. Whilst liquid chromatography (LC) systems provide a highly sensitive detection method for targeted species, gas chromatography, especially when coupled to mass spectrometry, is considered preferable for the screening of a wider range of target and unknown species. Existing chromatography methods are not without their limitations, not least the difficulty in reproducibly derivatising the wide range of steroids that can be exogenously administered, in particular the so called ‘designer steroids’ which deliberately hinder this process.

Initially, a pilot study was conducted to investigate whether aptamer-coated magnetic beads could provide a simple, low cost method for the extraction of estradiol from urine, prior to its detection and quantification with mass spectrometry. In reality, the process necessitated the use of additional solvent extraction steps to the standard method applied previously. These additional steps added complexity and were not without issue and the limit of detection was not found to be adequate for the proposed clinical purpose.

The project explored the application of comprehensive gas chromatography/mass spectrometry (GC x GC/MS) coupled to bespoke molecular imprinted polymers (MIPS) as an alternative solution to the problem of the screening of multiple steroids in urine. Twelve structurally similar and commercially relevant steroids were used to develop the chromatographic method. Once optimised, these compounds were then extracted from synthetic urine using a MIP templated with testosterone and developed during the project. Significant improvements compared to commercially available C-18 materials were found. All the steroids were identified and detected when spiked at the 10 ng/ml level, as required by World Anti-Doping Agency (WADA) at the time. The method was further validated and then successfully applied to the extraction of designer steroids from synthetic urine, in the presence of the original steroids. Epistane, which has a different structure to testosterone, was not detected indicating the efficacy of the templating process.

Acknowledgements

The author acknowledges with sincere gratitude the funding provided by The Open University and Anatune Ltd. The author will also like to thank HFL for providing the relevant steroid for analysis.

It is indeed difficult for me to describe in words my appreciation for my primary supervisor Dr. Geraint Morgan for his words of encouragement in times of need and unwithering belief and support throughout this project. I hope and pray that I have done justice to what I always believed was a once in a life time opportunity. This thesis carries my journey through crossroads, turns and to far distant mountains. The landscape from the top is beautiful but what carried me was the support of many: my co-supervisor Dr Maria Velasco-Garcia, and co-author, Dr. Nicholas Turner. My dear colleagues: Bill Guthery, Imran Janmohammed and Andrew Morris. The dreams and prayers of my mother Shaista Zulfiqar and the adoration for my late father Ahmed Zulfiqar who taught me to never give up. To my lovely wife Ambreen, thank you for being there for me.

My time at The Open University has taught me that the real opportunities in life are those in which you help and support others so I would like to thank a few people by name and extend this wider to everybody at The Open University for their help and support. A special thanks to Diane Turner for training me in the LECO TOFMS. Dr Youseff Elaziz for his help with the mass fragmentation patterns of steroids. Dr. Mabs Gilmour for all her help and laboratory support. The staff and atmosphere at The Open University has been truly amazing for me The Open University was more than an institute, it was home and I will miss it.

“One can only bring good intentions and dedication, success comes from God” Imran khan

Nomenclature

1D	one dimension
2D	two dimensions
1D-GC	one dimensional gas chromatography
µg	micrograms
µL	microlitre
δ ¹³ C	carbon isotopic composition
AAS	androgenic anabolic steroid
AE	appearance energy
APCI	atmospheric pressure chemical ionisation
CI	chemical ionisation
CRM	charge residue model
Da	Dalton
eV	electron volt
EI	electron ionisation
ELISA	enzyme-linked immuno sorbent assay
ESI	electrospray ionisation
FID	flame ionisation detector
FIFA	Fédération Internationale de Football Association
fg	femtogram
GC	gas chromatography
GCxGC	two-dimensional gas chromatography
GC-C-IRMS	gas chromatography-combustion-isotope ratio mass spectrometry
GC-FID	gas chromatography-flame ionisation detector
GLC	gas-liquid chromatography

HPLC	high performance liquid chromatography
hr	hour
i.d.	internal diameter
IEM	ion evaporation model
IRMS	isotope ratio mass spectrometer
kV	kilovolts
L	litre
LC-MS	liquid chromatography-mass spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantitation
m	metre
min	minute(s)
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption ionisation
mg	milligram
mL	millilitre
mm	millimetre
ms	millisecond
MS	mass spectrometer
MS/MS	tandem mass spectrometry or mass transition
ng	nanogram
NIST	National Institute of Standards and Technology
PDB	Pee Dee Belemnite
pg	picogram

PTV	programmable temperature vaporisation
SELEX	systematic evolution of ligands by exponential enrichment
SIM	selective ion monitoring
s/n	signal to noise
SPE	solid phase extraction
SRM	selected reaction monitoring
TMS	trimethylsilyl
UHP	ultra high purity
UPLC	ultra performance liquid chromatography
VPDB	Vienna Pee Dee Belemnite
WADA	World Anti-Doping Agency

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1. Introduction

1.1. Background: A brief history of sports testing

Sport, in all its forms, tends to bring out the competitive nature in men and women. For some athletes the desire to beat the competition, for the financial or social benefits resulting from success, can result in them using artificial means to enhance their performance.¹

The testing of human athletes for drug abuse began in the 1950s, when the widespread use of amphetamine first became evident. However, it was not until the death of two cyclists, one in the 1960 Rome Olympic Games and the other in the 1967 Tour de France, that things began to accelerate.² Experimental testing procedures were first employed in the 1968 Mexico City Olympic Games and official testing began at the 1972 Munich Olympic Games, where gas chromatography (GC) was first used with nitrogen-selective detection. Gas chromatography/mass spectrometry (GC/MS) was first employed at the 1976 Montreal Olympic Games and has been the main diagnostic method used since.³

Anabolic agents are the most common performance enhancing substances detected in athletes.⁴ The International Olympic Committee (IOC) banned the use of anabolic steroids in April 1974 and the World Anti-Doping Agency (WADA) was set up in 1999 with a view that two of its key activities should include the development of anti-doping capabilities and the monitoring of the World Anti-Doping Code - a document harmonizing anti-doping policies in all sports and all countries.⁵ By 2007, 223,898 samples were analysed by 33 WADA accredited laboratories worldwide; there were 4,402 Total Findings (Adverse Analytical Findings and Atypical Findings), of which 2,269 were for the use of anabolic steroids.⁶ By 2011, this had increased slightly to 4,856 Total Findings from 243,193 samples. Anabolic agents accounted for 59.4 % of the prohibited substances identified in reported findings.⁷

The number of drug tests conducted between 2005 and 2012 increased by 84,317; whilst, the percentage of adverse analytical finding has remained consistent between 1.1 to 2.0 %. The reported adverse analytical findings for anabolic steroids between 2005 and 2012 ranged from 715 to 1038. These figures show that the increasing number of doping tests has not resulted in a significant increase in the number of adverse analytical findings, suggesting the current WADA methods have limitations. These include the detection of athletes using AAS on a low-dose and the differing metabolic profiles of the tested individuals making their detection difficult. Such limitations may result in undetected doping samples in both in-competition and out-of-competition doping control programmes.^{8,9}

The field of sports testing is always changing with new synthetic anabolic agents being developed and applied. Given the large range in anabolic agents available, their structural similarities and the fact that trace level analyses are being performed in complex matrices, it is not surprising that no single analytical solution or technology has been developed, or is likely to be developed, that is capable of screening all anabolic agents. It is proposed in this thesis that comprehensive chromatography coupled to time-of-flight mass spectrometry and an updated sampling technique will provide an additional tool to the analysts, which could be used to improve the analysis and screening of steroids contaminants.

1.1.1. Anabolic androgenic steroids (AAS)

Anabolic androgenic steroids (AAS) are compounds that have the fused-ring structure of steroids and generate anabolic and androgen effects similar to those of testosterone. The anabolic effects of androgens include: accelerated growth of muscle, bone density, and red blood cells. The androgenic effects include changes in sexual characteristics, such as:

maturation of the penis and testes, voice changes, hair growth on the face, and increased aggressiveness. Since the anabolic effect can't be completely separated from the androgenic effect they are known as anabolic androgenic steroids.¹⁰

Testosterone is a natural androgenic steroid hormone produced by the testis and is ultimately derived from cholesterol.¹¹ As with all hormones, testosterone is a messenger molecule that travels through the body until it is either metabolised or interacts with cells containing androgen receptors. The mechanism for the biosynthetic formation of testosterone from cholesterol is shown in Figure 1.1.¹¹

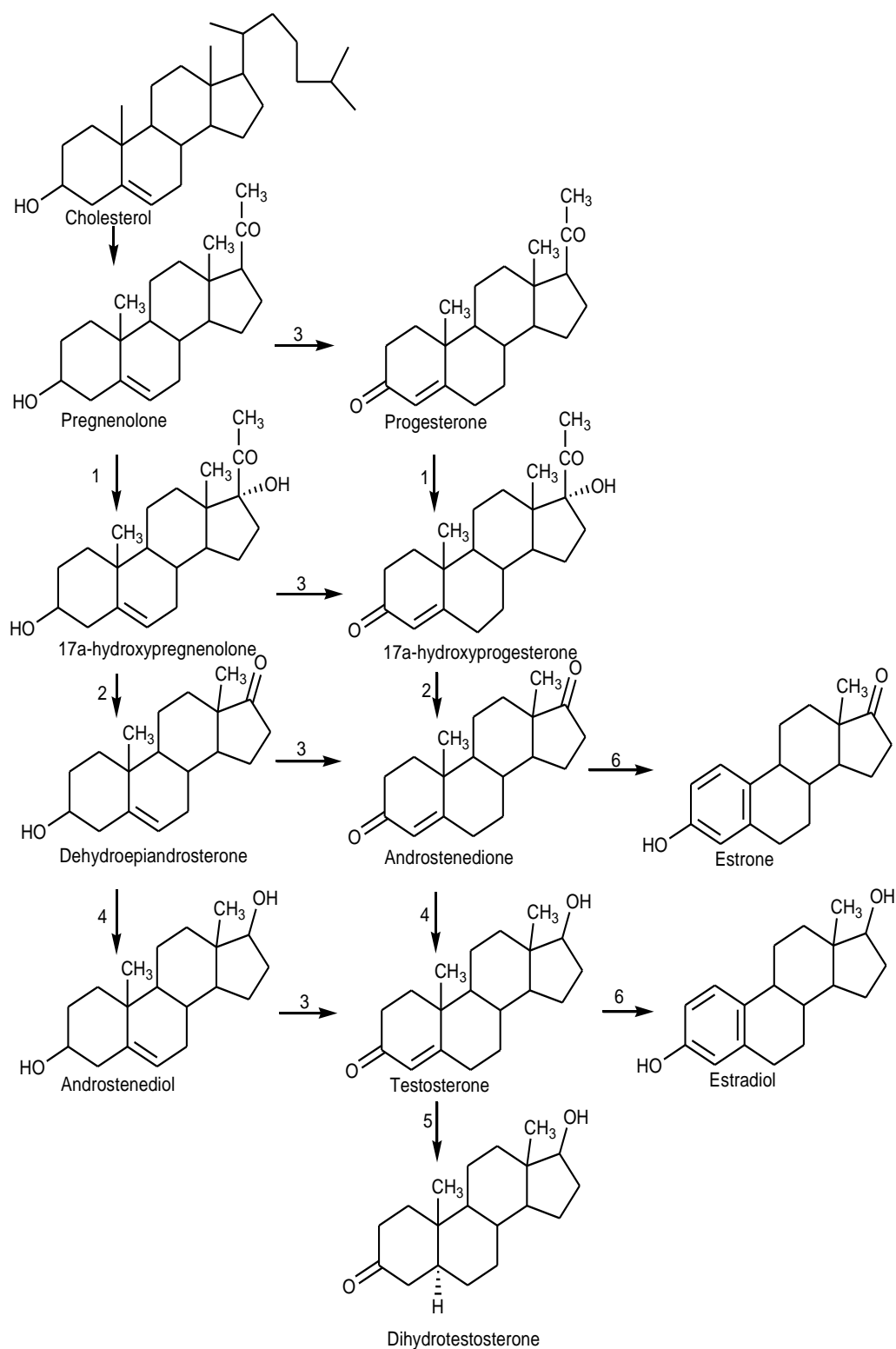


Figure 1.1. The mechanism for the formation of testosterone and estrogens from cholesterol, via progestogens and androgens, with 1-6 being a series of enzymes.

As can be seen in the figure, the conversion of cholesterol to testosterone proceeds via a number of progestogens (21 carbons) and androgens (19 carbons). Cholesterol itself is derived from lanosterol which, in turn, is formed from the cyclisation of squalene. Each of the steps in the formation of testosterone is catalysed by a series of enzymes, including: 17 α -hydroxylase [1]; 17,20 lyase [2], 3 β -hydroxysteroid dehydrogenase (3 β -HSD) [3], 17 β -hydroxysteroid dehydrogenase (17 β -HSD) [4].¹¹ Testosterone, in turn, is converted to: (a) dihydrotestosterone (DHT) by 5 α -reductase [5], contributing to male pattern baldness, prostate growth and sebaceous gland activity; or to (b) estradiol, through aromatase enzymes [6]. The primary pathway for the formation of estradiol is via estrone and 4-androstenedione.

Synthetic AAS are structurally modified to maintain the anabolic effect while reducing the androgenic effect. The use of anabolic steroids was first attributed to weightlifters in 1954 and by 1965 synthetic AAS were being used by bodybuilders and weightlifters.¹²

AAS stimulate protein synthesis in muscles cells allowing greater lean mass.¹³ The dose of the anabolic steroids taken is determined by the sports and the needs of the athletes. For example, endurance athletes use steroids primarily for the catabolism-blocking effect which prevents breakdown of muscles during intense exercise.^{3,12} Sprinters administer slightly higher dosages because of the strength and power required in the sport. This dosage can be almost double the natural replacement level.³ However, in sports requiring athletes to bulk up their muscle mass, such as bodybuilding, athletes will take dosages 10 to 100 times the normal physiological level.³ A theory underlining the high dosage effect of AAS suggests that glucocorticoids that bind to glucocorticoid receptors are displaced by a high dosage of AAS resulting in an anti-catabolic effect.¹⁴

Anabolic steroids are often taken in cycles of 6 or 12 weeks. Athletes will traditionally take more than one steroid, referred to as “stacking”. Stacking allows users to activate more receptor sites or to achieve a synergistic effect, *i.e.* the presence of one steroid enhancing the effect of the other.^{3,12,15} Steroid users will usually begin with a lower dose, progressing to a higher dose, and then reducing the dosage at the end of the cycle. Athletes will also indulge in a number of other drugs, to counteract the side effects of the initial drug, these include stimulants, diuretics, anti-estrogens and anti-inflammatories.^{3,15}

1.1.1.1. The structure of anabolic androgenic steroids

The structure of AASs are based around the sterane nucleus. As shown in Figure 1.2, it consists of three condensed cyclohexane rings (A, B and C) and a cyclopentane ring (D).

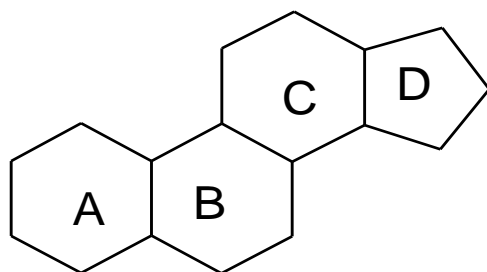


Figure 1.2. The sterane nucleus.

A number of modifications can be made to structures including the introduction of:^{3,9,13}

- carbonyl groups,
- hydroxyl groups,
- double bonds,
- other substituents (such as alkyl groups),
- ring structure changes

1.1.1.2. Steroid nomenclature

There are a number of different methods for naming steroids, one such convention is:

- prefix and suffix indicate presence of substituent,
- “nor” is prefix to indicate the elimination of a methyl group,
- if there is more than one prefix then they are written in alphabetic order,
- the position of double bonds is indicated by numeration of the lesser carbon atom,
- substituents below the plane are denoted as α and those above the plane are denoted as β ; in this thesis they are denoted as a and b, respectively.

The numbering of the carbon atoms in steran nucleus is shown in Figure 1.3.¹⁶ The simplest steroid is gonane, which only has hydrogen attached to the seventeen carbon fused-ring nucleus.

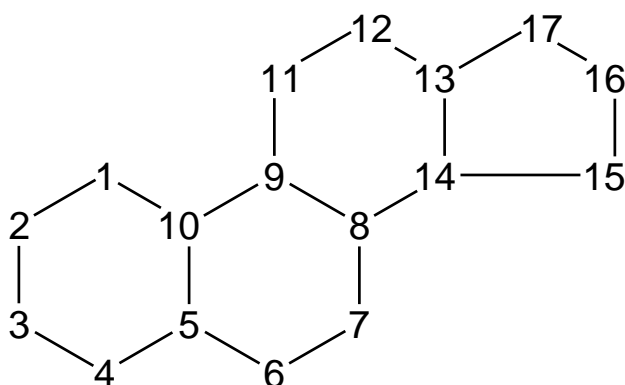


Figure 1.3. The numbering convention for carbon atoms in the steran nucleus.

1.1.1.3. The biological properties of anabolic androgenic steroids

When testosterone was isolated in 1935, it was soon realised that it was inactive when taken orally, as it is transported to the liver and is metabolised to an inactive form. However, a structural modification in testosterone has been shown to change its biological activity, while its androgenic and anabolic actions are also influenced.^{14,15,16} For example, the

androgenic effect in testosterone is mainly due to the presence of the 17 β -hydroxyl group and according to Gower *et al.*, the androgenic activity is completely lost by the removal of this group.¹⁵ Oxidation of the 17 β -hydroxyl group can also affect the androgenic activity and a reduction in androgenic activity is also observed following the introduction of an additional double bond into Ring A.¹⁷

Analysis of the structure of natural steroids has led to the synthesis of many artificial hormones. Structural modification of testosterone, as shown in Figure 1.4, aims to increase the anabolic effect, reduce the metabolism of AAS and allow a lower dosage.^{15,16}

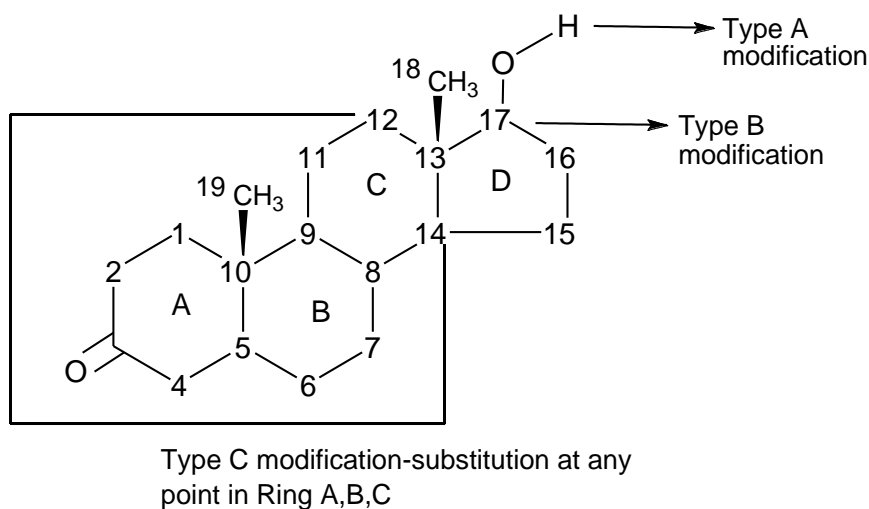


Figure 1.4. The three major types of modification of testosterone.

As stated previously, Gower *et al.*, showed that a Type B modification of the hydroxyl group in the 17-position results in complete loss of androgenic activity, whereas Type A modification can also affect the androgenic activity.¹⁷

The reduction in the metabolism rate is achieved by slowing the release of AAS from the muscles into the blood. Type C modification of testosterone can result in the synthesis of nandrolone (19-nortestosterone) which contains no methyl group in the 19-position, as shown in Figure 1.5. The loss of the methyl group results in it being released into the blood more slowly than testosterone.¹⁸

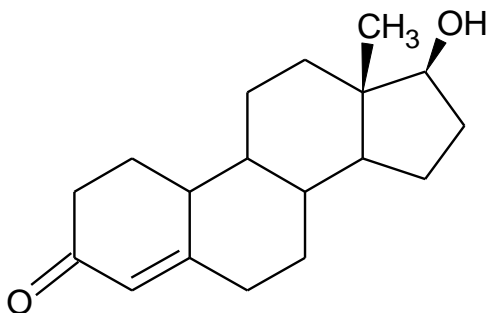


Figure 1.5. The structure of nandrolone.

1.1.1.4. The metabolism of steroids - Phase I reactions

Steroids are rapidly metabolised and can only be detected in their original state for a short period of time. The study of steroid metabolites, which remain longer in the excreted urine, is therefore very important in doping analysis.^{15,17,19} The metabolism of steroids may be divided into two phases: Phase I and Phase II.

In Phase I reactions, the AAS are converted to more polar compounds to facilitate their elimination from the body. Phase I catalyzed reactions involve oxidation, reduction and hydroxylation of rings A, B, C and D.²⁰

In the metabolism of Ring A, the reduction of the C4-C5 double bond is catalysed by the 5a- and the 5b-reductase, located in the liver, resulting in 5a- and 5b-isomers, as shown in Figure 1.6.^{20,21} The structure of the steroids ultimately determines the metabolic pathway, as structural modification prevents certain metabolic pathways. AAS, such as metandienone

do not produce 5a-isomers.^{21,22} Depending on what substituents are present in Ring A, other metabolites may also be formed.^{20,23}

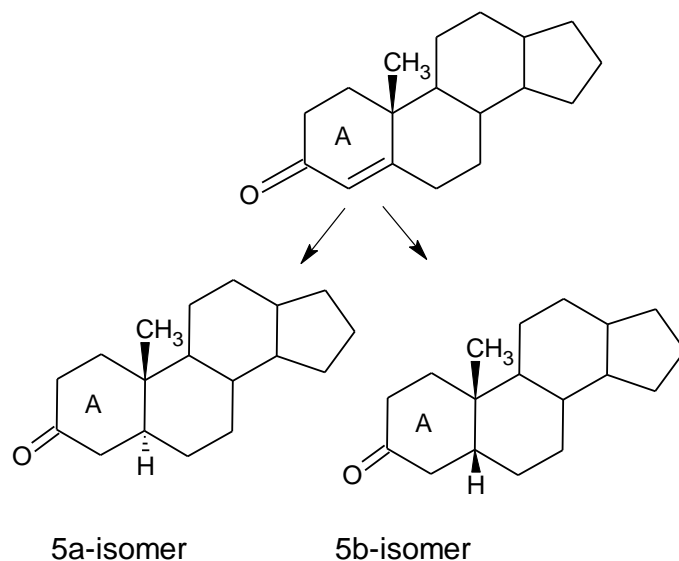


Figure 1.6. Metabolism of Ring A, catalysed by 5a- and 5b-reductase.

Ring B metabolism is more pronounced in AAS that have two double bonds present in Ring A. The main metabolic reaction is the beta-hydroxylation at C6, as shown in Figure 1.7.^{20, 24,25}

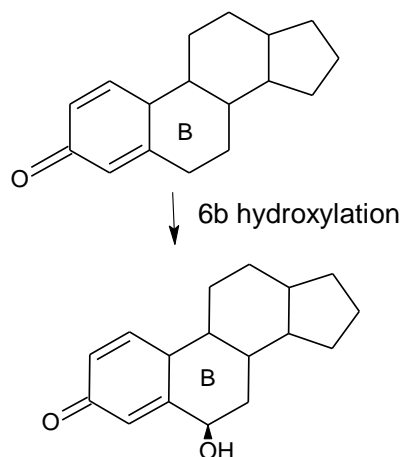


Figure 1.7. An example of Ring B metabolism.

Metabolism in Ring C is not common; however, a hydroxylation at position 12 in Ring C takes place for the steroid metandienone, as shown in Figure 1.8.^{20,25}

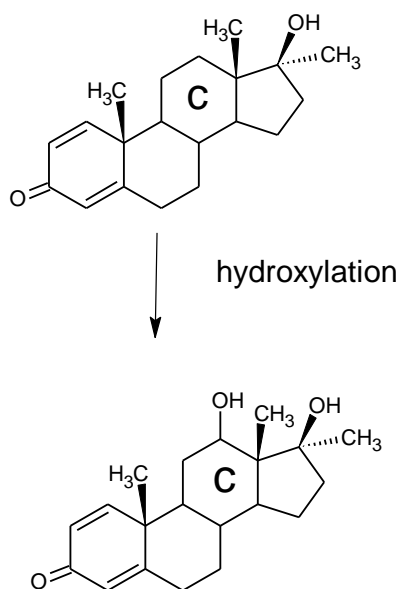


Figure 1.8. An example of Ring C metabolism.

In Ring D hydroxylation, the most common reaction is at the 17b-position. This reaction is catalysed by the enzyme 17b-hydroxysteroid dehydrogenase (HSD) forming a 17-keto steroid, as shown in Figure 1.9.²⁶ However, this reaction is reversible and depends on other metabolic steps. Steroids containing a 17-hydroxyl group are mainly excreted as a 17-keto metabolite. Hydroxylation can also take place at position 16 resulting in 16a- and 16b-isomers. This oxidation is also shown in horses administered with stanozolol.²³

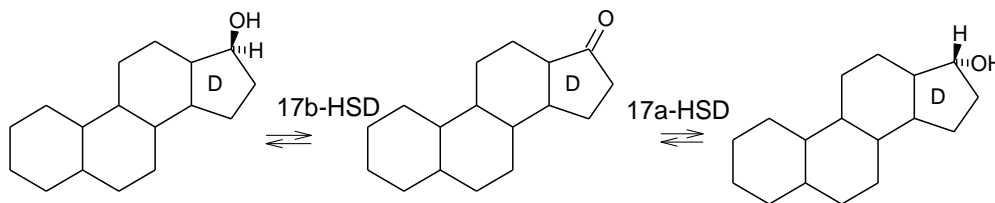


Figure 1.9. An example of Ring D metabolism.

1.1.1.5. The metabolism of steroids - Phase II Reactions

Phase II reactions involve conjugation of AAS with glucuronic acid or sulphonic acid, as shown in Figure 1.10. Conjugation assists the elimination of the steroids from the body in urine, through an increase in the hydrophilic nature of the steroid.¹⁹ Conjugation in the A ring occurs at the 3-position with the 3a-hydroxyl group conjugating with glucuronic acid, while the 3b-hydroxyl group conjugates with sulphonic acid.²⁶ Phase II reactions also occur prominently in the D ring. Glucuronidation occurs at the 17b-hydroxyl group; however, it is important to point out that in 17a-methyl steroids, glucuronidation does not occur because of steric hindrance. However, sulfate conjugates do form and the reaction was first described for metandienone administration in horses.²⁷

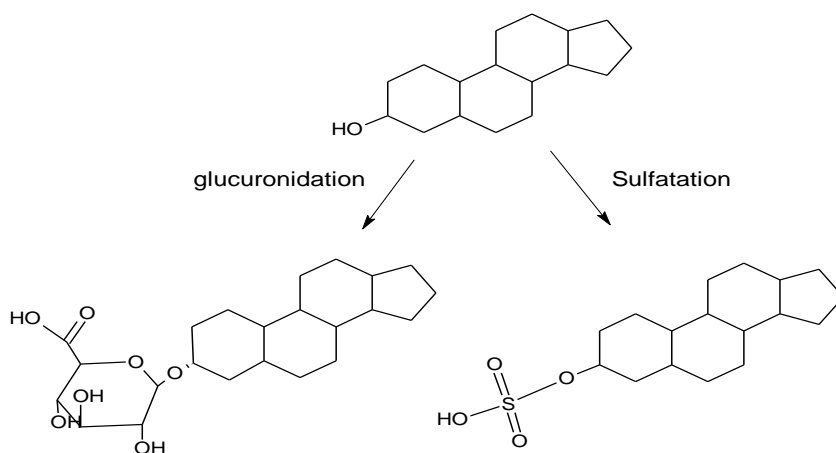


Figure 1.10. Conjugation reactions of Ring A, with either a glucuronide or sulfate moiety.

1.1.2. World Anti-Doping Agency (WADA) Prohibited List

The World Anti-Doping Agency (WADA) is the independent international body responsible for coordinating and monitoring the global fight against doping in sport.⁵ Established in 1999, it has evolved into a global network of committed sports and government authorities and individuals working together to protect athlete health and the

integrity of sport. WADA publishes a Prohibited List, which, in 2007, defined prohibited substances grouped into the following categories:²⁸

S1. Anabolic androgenic steroids (AAS)

- exogenous and endogenous

S2. Hormones and related substances

S3. Beta-2 agonists

S4. Agents with anti-estrogenic activity

S5. Diuretics and other masking agents

S6. Stimulants

S7. Narcotics

S8. Cannabinoids

S9. Glucocorticosteroids

In the context of this thesis we will only consider the analysis of anabolic androgenic steroids (S1).

1.1.2.1. Anabolic androgenic steroids (S1)

WADA publish, and regularly update, a list of prohibited endogenous and exogenous AAS. The latest list includes forty six exogenous compounds named specifically, along with the statement that other substances with similar chemical structure or similar biological effect(s) should also be considered prohibited.

Endogenous AAS (when administered exogenously) include the androgens shown in Figure 1.1, namely: androstenediol, androstenedione, dihydrotestosterone, DHEA, and testosterone

and their metabolites and isomers. A list of twenty two such species is provided along with the statement that substances considered prohibited are not just limited to this list.

Samples are considered to contain prohibited substances:

- if the concentration of such prohibited substance in the athlete's sample is above typical range of values normally found,
- if the laboratory can show that the prohibited substance is of exogenous origin,
- if the testosterone to epitestosterone (T/E) ratio is equal to or greater than 4.0.

A simple threshold value in the urine sample cannot be used to prove exogenous administration of steroids that are produced endogenously, such as testosterone. This is because levels of such compounds can vary considerably in athletes. However, the steroid profile of testosterone glucuronide and its minor metabolite epitestosterone glucuronide (T/E ratio) should remain fairly constant in the urine excreted. Exogenous application of testosterone will result in an increase in the urinary concentration of testosterone glucuronide; however, the level of epitestosterone glucuronide will remain the same. In 1983, the International Olympic Committee (IOC) adopted a T/E ratio of 6.0 as the threshold for constituting an offence. In doping analysis, if the T/E ratio was found to be greater than 6.0, longitudinal studies were undertaken to establish if the ratio was high due to physiological or pathological conditions. The athlete's individual T/E reference value was established, the concentration of testosterone and epitestosterone and the concentration of major metabolites were also determined. In 2004, the guidance on this T/E ratio was changed.²⁹ Any sample with a T/E ratio of > 4.0, or if concentrations are greater than the set limits for testosterone, epitestosterone, androsterone, etiocholanolone or DHEA then the urine sample should be submitted to gas chromatography/combustion/isotope ratio mass

spectroscopy (GC/C/IRMS). By using GC/C/IRMS, stable carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) allow discrimination of endogenous and exogenous steroids, the exogenous sources containing less ^{13}C than their endogenous homologues.

The basis of GC/C/IRMS, is that stable carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) found in organic molecules show very small variations due to the processes and feed stocks involved in their production; this measurable variability can be used to distinguish between endogenous and exogenous sources of steroids. The carbon isotopic composition of a sample is expressed as $\delta^{13}\text{C}$, is measured in units of per mil (‰) and is defined by the Urey equation (Equation 1):

$$\delta^{13}\text{C}(\text{‰}) = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{SAMPLE}} - (^{13}\text{C}/^{12}\text{C})_{\text{STANDARD}}}{(^{13}\text{C}/^{12}\text{C})_{\text{STANDARD}}} \times 1000$$

Equation 1. Urey equation for determining the stable isotopic composition of carbon

The δ symbol is standard notation and refers to the isotopic shift against a known reference standard. In the case of carbon the international reference standard was a Pee Dee Belemnite (PDB) carbonate, which has now been replaced by a new standard, Vienna Pee Dee Belemnite (VPDB).³⁰

Exogenous testosterone contains comparatively less ^{13}C stable isotope than endogenous testosterone; this is because they are usually synthesised from soy, which is depleted in ^{13}C when compared to traditional dietary sources of cholesterol. In the technical WADA documentation, adverse analytical findings are reported when the $\delta^{13}\text{C}$ value is more than 3‰ away from the value obtained from the chosen urinary reference steroids.²⁹

It should be noted that a number of papers in the literature still debate the validity of the T/E>4.0 ratio and the required confirmatory test using gas chromatograph/combustion/isotope ratio mass spectroscopy (GC/C/IRMS).^{31,32,33}

In one study, it was shown that shifts in $\delta^{13}\text{C}$ can be caused by the length of time before urine samples are tested, as well as the length of time a subject stays in a different country.³¹ This two year longitudinal profile studied three caucasian male athletes that travelled between Africa and Europe, residing for one to two months in Africa, while the rest of the time was spent in Europe. Results showed enrichment of $\delta^{13}\text{C}$ after a subject was tested within two weeks of arrival in Europe. However, when a subject was tested one or two months after arrival he did not show any $\delta^{13}\text{C}$ enrichment. The length of stay in Africa also affected the enrichment of $\delta^{13}\text{C}$.

Urine samples collected from African countries were found to have higher $\delta^{13}\text{C}$ content because of diet. Ultimately, the $\delta^{13}\text{C}$ in the African population is linked to the eating habits in the region. The higher consumption of maize and sugar cane in Africa will result in an enriched $\delta^{13}\text{C}$ value for endogenous steroids, as they are produced from cholesterol, which is derived from plant tissue and will be dependent on the photosynthetic pathway of the plant consumed.²⁹ It should be noted that the study did not find any false positive samples, as the range of $\delta^{13}\text{C}$ was within the isotopic variation allowed by WADA. However, recommendations were made to evaluate further.

In another case, when seven urine samples produced similar high testosterone to epitestosterone ratios of between 3.7 to 4.2, genetic profiling showed that three of the samples were from the same athlete. The results highlighted that the T/E ratio alone was not appropriate to identify individual urine samples.³⁴

Donike *et al.*, the pioneers of the current anti-doping procedure, have suggested that a population-based reference range is not suitable because of individual variations, since the athlete can have an undetected increase in the T/E ratio.³⁵ No formal procedure has yet been formulated for the interpretation of the time profile of the T/E ratio.³⁶

In one study, 520 urine samples were tested using the T/E ratio threshold of 4.0. The study returned 24 false positive and 34 true positives.³² The Bayesian interpretation of a statistical model used in this study detected a true positive result at T/E ratio of 2.1 and a false positive at T/E ratio of 14.2.³²

Furthermore, nine out of competition samples taken during and after the 2014 FIFA World Cup in Brazil, showed a T/E ratio higher than 4.0 and one in-competition sample had a T/E ratio of 4.1. However, when these samples were tested by GC-C-IRMS, to determine if the testosterone was exogenous, the result showed that all samples were false positives.

1.1.2.2. Urine analysis in sport testing

The screening of athletes is usually enacted through the analysis of urine samples at specialised, accredited laboratories.³⁷ The first stage of human urine testing is the collection of two samples, labelled A and B. This procedure is conducted by a sample collection officer and does not involve the testing house. The specific gravity of the urine is measured at this stage. The samples are uniquely bar-coded and sealed in tamper-proof packaging and an affidavit is signed by the athlete. Samples are then sent to the testing house. On arrival, the samples are logged into the laboratory information management system (LIMS). The specific gravity is re-measured and compared with the original results. If the two results are comparable then further analysis can take place. If, however, abnormal results are shown then the possibility of sample tampering or sample deterioration need to be addressed. The

B samples are stored; whereas, the A samples are divided into five aliquots, one for each of the following test methods:

Sample 1A: Peptide hormones: enzyme-linked immunosorbent assay (ELISA)

Sample 2A: Basic drug compounds, stimulants, narcotics and beta blockers:

Gas chromatography/mass spectrometry (GC/MS) in full scan mode

Sample 3A: Conjugated steroids: GC/MS and liquid chromatography/ tandem mass spectrometry (LC/MS/MS)

(GC/MS in selective ion monitoring (SIM) mode)

Sample 4A: Unconjugated steroids and diuretics: GC/MS & LC/MS/MS

Sample 5A: Designer steroids: LC/MS/MS

The first aliquot is tested for peptide hormones using enzyme-linked immunosorbent assay (ELISA). ELISA is applied to species not compatible with chromatographic methods, such as peptide hormones. The main process in ELISA is the binding of the antigens to antibodies, if present in the sample. The plate is then washed to remove all interferences. A secondary antibody is then applied to the plate, followed by another wash. This secondary antibody is linked to an enzyme and when a substrate is added it will lead to catalysis and produce a coloured product or a detectable change in colour or fluorescence.

The second aliquot, which tests for basic drug compounds, stimulants, narcotics and beta blockers is pH modified and an internal standard is added. Enzymatic hydrolysis of glucuronic acid conjugates is performed. Potential interferences are removed from the

sample via solid phase extraction, the eluent is then evaporated and reconstituted in a silylating mixture. The sample is screened by the GC/MS in full scan mode, using electron impact ionisation (EI) at 70 eV. An automated data processing system conducts a library search and detects peaks. A report is generated by the system which is then reviewed by an analyst.

The third aliquot, testing for conjugated steroids, is enzymatically hydrolysed after pH modification and addition of an internal marker. It is then extracted using a combination of SPE cartridges to give a neutral and basic fraction. The neutral fraction is derivatised by forming trimethylsilyl derivatives and analysed by GC/MS in selective ion monitoring (SIM) mode, with 30 different acquisition time windows, each containing between 10 and 15 ions. A report is automatically generated by the system. The ratio of testosterone to epitestosterone is also calculated by the automated procedure. The basic fraction is analysed by LC/MS/MS.

The fourth aliquot tests for unconjugated steroids and diuretics. It is pH modified, an internal marker is added and then extracted by SPE, before splitting it into two portions. The first portion being tested by GC/MS after trimethylsilyl derivatisation and the second portion tested by LC/MS for diuretics.

The final aliquot is used to test for difficult or problematic analytes, such as designer steroids which are specifically synthesized to avoid detection.

If samples generate a suspicious finding then confirmatory analysis is undertaken. As this data may be used in the court of arbitration for sport, the whole procedure is now repeated by a single analyst. Blank urine samples and reference material are also used for confirmatory evidence for the presence of steroid in the urine sample.³⁷

1.1.3. The contamination of nutritional supplements

Dietary supplements are often taken by athletes and the general public, and can include vitamins, minerals, herbs, and amino acids.³⁸ Up until 2002, dietary supplements were regulated as a class of foods, not as drugs and therefore were not screened for safety or efficacy. Ambiguity in the law allowed companies to exploit health-promoting properties of dietary supplements without regulatory obligations to display any known risks.

The following risks have been associated with dietary supplements:^{39,40}

- severe or acute side effects, *e.g.* kava with liver failure and aristolochic acid with kidney failure,
- side effects may be associated with prescription medicine,
- postponement or delay of conventional medical treatment,
- harmful interaction with prescription and over-the-counter medications.

The Financial Times reported that the global market for supplements, in 2001, was estimated at US\$46 billion and the US supplement market, in 2000, was estimated at US\$16.7 billion.⁴¹ In 2004, global sales were estimated to be between US\$75-250 billion, this large range was due to the differing definitions of a supplement from country to country, resulting in complex sales data.⁴² Nevertheless, the sales are expected to grow to US\$384 billion in 2014.⁴³ Factors influencing the growth of the supplement market include easy access through the internet, which makes it possible for users all over the world to access all products freely available in the USA and promotion of the use of supplements by top athletes. General population studies on the use of supplements show a 40 % rate of use.⁴⁴

These products are most frequently used by sportsmen and sportswomen, ranging from amateur level to elite athletes, the category to which they are most aggressively marketed.⁴⁵ The high prevalence of use can be illustrated by a study following the Olympic Games held in Sydney in 2000. In this study, the medication use of 2,758 athletes, selected for doping control, was evaluated.⁴⁶ The major findings were that 51 % of athletes tested had used vitamins before competition and a large fraction of the tested athletes used other nutritional supplements. Some athletes even reported using up to twenty-six different supplements. Commonly cited reasons for supplement use included:

- to compensate for an inadequate diet,
- to meet the abnormal demands of hard training,
- to benefit performance,
- to keep up with team-mates or opponents,
- recommended by coach, parent or other influential individual.

According to the 1994 Dietary Supplements Health and Education Act (DSHEA), nutritional supplements that did not claim to “diagnose, treat, prevent or cure disease” were not subject to regulation by the Food and Drug Administration (FDA).⁴⁷ The advantage for supplements companies was that there was neither a requirement to prove claimed benefits nor any legislation to show safety with acute or chronic administration. No quality assurance and or strict labelling requirements led to supplement contamination.

The FDA warned several manufacturers and distributors of unapproved drugs containing steroids and had stated that any further distribution or sale of these products without their approval would result in regulatory action, including seizure and injunction.⁴⁸ The FDA is primarily concerned that the use of such products, that are marketed as dietary supplements

and promoted for building muscle and increasing strength, may cause serious long-term adverse health consequences in men, women, and children. These products claim to be anabolic and problems associated with anabolic steroids include: liver toxicity, testicular atrophy and male infertility, masculinisation of women, breast enlargement in males, short stature in children, adverse effects on blood lipid levels, and a potential to increase the risk of heart attack and stroke.

The FDA have also recalled products containing excessive doses of vitamins A, D, B6 and selenium because of potentially toxic effects. Some products have been shown to contain impurities (lead, broken glass, animal faeces, *etc.*) because of poor manufacturing practice.⁴⁹

Although the primary goal of The Dietary Supplement Health and Education Act was to ensure consumers had easy access to diverse dietary supplements such as vitamins, it inadvertently resulted in several new steroids being introduced as nutritional supplements.^{50,51} Initially, these new steroids were precursors of testosterone and are therefore collectively referred to as prohormones.⁵² These hormone precursors have limited anabolic or androgenic properties themselves but are intended to be converted to fully active hormones, via the enzymatic process that occur during metabolism, typically resulting in the addition of whichever atoms happen to be missing from the chemical structure of the compound.

In 1996, the first steroid introduced on the prohormone market was dehydroepiandrosterone (DHEA).⁵³ When taken exogenously, this endogenous steroid was classified as a prohormone, as it was advertised to metabolise to testosterone after oral administration. Androstenediol, androstanediol and 19 Nor- analogues emerged at the same time. These

prohormones are thought to be converted in the body to testosterone and nandrolone, respectively. Boldine is also used as a prohormone for boldenone. Some prohormones have even been disguised as nutritional supplements, thus avoiding statutory control.⁵⁴

1.1.3.1. Nutritional supplement testing

Geyer *et al.*, have conducted three major studies on the contamination of nutritional supplements.⁵⁵⁻⁵⁷ Their initial analysis, published in January 2000, found contaminated nutritional supplements in three products. They conducted a second study, on a further 153 nutritional supplements, followed by an international study of 634 samples.

In the first study, at the International Olympic Committee (IOC) accredited laboratory in Cologne, they reported the results of an analysis carried out on dietary supplements, none of which declared on the label that they contained steroids.⁵⁵ The nutritional supplements tested had been produced in the USA. Nandrolone, testosterone and other steroids were identified in these supplements. When they were fed to healthy volunteers, they resulted in positive test results, *i.e.* enough steroids in the urine to result in a failure in a drug test.

The total amount of steroids varied between 0.3 µg and 5,000 µg per capsule. After administration of one capsule of the above mentioned nutritional supplements, all volunteers showed positive results for the nandrolone metabolite, norandrosterone. Three to four hours after administration of the capsules, urinary concentrations of between 4 and 623 ng/mL were measured for norandrosterone. A female volunteer additionally showed an increase in the ratio of testosterone/epitestosterone from 0.6 to 4.2.⁵⁵

The laboratory followed this up with the analysis of 153 supplements bought in the USA, UK, Sweden, Norway, Germany, Belgium, Israel and Austria.⁵⁶ The study showed:

- 18 different supplements (from 12 different companies) contained prohormones that had not been declared on the label,
- 15 of the 18 supplements contained 19-norsteroids,
- 19-nor-4-androstene-3,17-dione and 4-androstene-3,17-dione were the most commonly found prohormones in the contaminated nutritional supplements.

Contaminated nutritional supplements included carnitine, vitamins, minerals, Tribulus Terrestris, ribose, branched chain amino acids, ornithine keto-glutarate, creatine, zinc, pyruvate, chrysin, guarana, conjugated linoleic acid, herbal extracts and glutamine.

The extended analysis showed a number of disturbing findings. About 50 % of the analysed supplements showed a concentration of prohormones higher than 10 µg. The maximum detected was 5,140 µg. More than 10 % of the analysed supplements contained prohormones not declared on the label. The results showed that the amount of prohormones were much lower than the amount usually present in a prohormone product. Results also showed a sharp variation from capsules to capsules, which led the author to conclude that the prohormones had been introduced through cross contamination. Further evidence was provided by the fact that 4 out 12 (33 %) of the non-hormonal supplements that were found to be contaminated came from companies that also sold prohormones. Interestingly, 14 of the contaminated nutritional supplements did not come from companies that sold prohormones.

Geyer *et al.*, also quantified the amount of steroids needed to cause positive doping results. They concluded that 1000 µg/capsule of 19-norsteroids would cause a positive doping results for norandrosterone for more than 24 hours. However, they also showed that 19-norandrosterone was still in the urine at a concentration of 2 ng/mL (WADA threshold)

four hours after the application of only 0.8 µg of norandrostenedione.⁵⁶ More recently, separate studies by Watson and colleagues and van der Merwe *et al.*, have shown similar results for dietary supplements.^{58,59}

Geyer *et al.*, then conducted a comprehensive international study of 634 samples from 13 countries and from 215 different suppliers.⁵⁷ The study was conducted between October 2000 and November 2001, with 289 supplements obtained from companies supplying prohormones and 345 supplements from companies not selling prohormones.

Results showed that 94 samples were contaminated with prohormones:

- 23 samples contained prohormones of nandrolone and testosterone,
- 64 samples contained prohormones of nandrolone only,
- 7 samples contained prohormones of testosterone only.

The distribution in the concentration of contaminated steroids varied from the study conducted earlier:

- only 16 % of the samples had a concentration above 10 µg. (compared to above 50% in the earlier study),
- most significantly, 75.5 % supplements had contamination levels below 5 µg,
- 56 % were below 2 µg - in fact 28.7 % were even below 0.2 µg.

In this study, it was declared that capsules had the most significant contamination, followed by tablets and powders. Substantial numbers of positive tests were obtained from products bought in the Netherlands (26 %), the USA (19 %), the UK (19 %) and elsewhere. The names of the contaminated supplements have not been published, but they included vitamins and minerals, protein and amino acid supplements, creatine and many others.

In 2007, the IOC-accredited laboratory in Vienna published a study of 57 supplements. Parr *et al.*, found that 12 (22 %) of these contained prohibited steroids.⁶⁰ The identities of the companies and the products have been published. As in the Geyer *et al.* study the presence of anabolic androgenic steroids was assumed to be from contamination in the manufacturing, or perhaps the distribution process. The reason given was that the contaminants were only present at low levels and were variable between and within batches.

In 2002, however, Geyer *et al.*, found alarming concentrations of the androgenic anabolic steroid metandienone in three nutritional supplements.⁶¹ These three supplements were bought in England, via telephone order. Metandienone was present in very high amounts, an average of 17.30 mg/g in one supplement. The highest concentration of metandienone found in a single capsule was 28.9 mg/g. This is more than enough to produce serious side-effects. Metandienone is a prescription only medicine. The therapeutic dose is 5-10 mg per day. If one was to consume the three capsules per day, as recommended by the manufacturer, this would result in metandienone dosages that were supra-therapeutic. Side effects of metandienone include abnormal liver function and physiological disorders. Long term application can lead to dependence and severe withdrawal symptoms. It has been alleged that this was a purposeful and deliberate contamination.

Nutritional supplements have an important position in the consumer market. The need for regulator intervention was imperative, as the danger for consumers exposed to harmful substance is real. The most comprehensive worldwide study undertaken of 634 samples had shown 94 samples to be contaminated with anabolic androgenic steroids.⁵⁹

In 2004, the US Congress amended the Anabolic Steroid Control Act, which resulted in the restricting of the sale of anabolic steroids and prohormones as nutritional supplements.⁶²

The act stated: “The term 'anabolic steroid' means any drug or hormonal substance, chemically and pharmacologically related to testosterone (other than estrogens, progestins, corticosteroids and dehydroepiandrosterone).” It also listed seventeen compounds as prohormones, along with their salt, ester or ether. The list was not closed and therefore any compound that affects testosterone is now considered prohibited under US law.

Although the history of nutritional supplement remaining entwined with DSHEA legislation, the regulation of nutritional supplements remains complex within the European Union. The difficulty is in distinguishing food supplements from medicinal products and in implementing this legislation across the union.

The nutritional supplement market is driven by consumer demands and behaviour in individual state country; for example, garlic is sold in the UK for cold treatment, whereas in Germany it is sold for the treatment of arteriosclerosis.⁶³ The consumption of a nutritional supplement is therefore often determined by an individual's perception of the perceived benefit. A balance therefore needs to be found between protecting consumers from over consumption, or contamination, and at the same time providing freedom of choice.

Under EU legislation it is illegal for a food product to make a statement regarding treatment and cure of a disease. Food Supplements are defined in Article 2 of 2002/46/EC Food Directive. The European Food Safety Authority (EFSA) defines a food supplement as a “sources of nutrient or other substance with a concentrated source”.^{63,64}

In the UK the Government Chemist provides guidance on the classification of supplements as food or medicinal products.⁶⁵ The guidance covers the latest UK and European legislation and also provides a decision tree to enable a classification to be made. One of the most important questions asked is “can it be administered to humans for restoring, correcting or modifying physiological function?” If the answer is yes, then the product is defined as a medicinal product and the Medicines Act (1990) applies; if the answer is no, then the product is a food and either the Food Safety Act 1990 or the Food Supplement (England) Regulations 2003(1) apply.⁶⁵

The Food Standard Agency and the Medicine Healthcare product Regulatory Agency (MHRA) monitor products within the UK.⁶⁵ The Rapid Alert System in Food and Feed (RASFF) portal allows European Union member states to flag and register adverse contaminated nutritional products, thereby allowing a rapid response.⁶⁶

In view of the challenges facing any athlete, HFL Sport Science have developed the Informed-Choice Programme to help customers distinguish between established and rogue nutritional supplements practises.⁶⁷ Their quality assurance program for sports nutrition products certifies that all nutritional supplements and/or ingredients with the Informed-Choice logo have been tested for banned substances.⁶⁷

Whilst legislation has been introduced to try to combat the problem with steroids and prohormones, based on the wealth of analytical evidence, several issues with the methods utilised were raised during the large-scale studies. In the extensive Geyer *et al.* study, 66 samples could not produce reliable results. All testing laboratories are facing a similar problem with a number of their samples have produced inconclusive results and is mainly attributed to matrix effects.

It is clear from this brief review of the literature that sports testing, both in terms of deliberate and inadvertent administration, is a highly dynamic field that is constantly changing with trace level analyses being performed in complex matrices. Before we explore the hypothesis that comprehensive chromatography, coupled to time-of-flight mass spectrometry and new sample extraction techniques could provide a valuable additional tool to improve the screening of steroids contaminants, we will briefly review the techniques currently in use and to be applied in this thesis.

1.2. A review of the analytical techniques applied in this thesis

1.2.1. Chromatographic separation

Chromatography is derived from two Greek words, meaning colour and to write, and its verbatim translation is “colour writing”. The impact of chromatographic separation has been known about since antiquity – the idea that a mobile phase (*e.g.* liquid) could separate a mixture of compounds (*e.g.* different coloured dyes) when passed along a stationary medium (*e.g.* cloth or paper) has been utilised extensively over the ages. Prof. Leslie Ettore, stated that the word was first used in its current scientific context in a publication by Mikhail Tswett, in the German Botanical Society, in 1905; although, he had first described the separation of plant pigments, by liquid chromatography (LC) columns, in a lecture in 1903.⁶⁸ Separation was achieved in a glass column; however, initially there was little recognition of the botanist’s work.

Further major developments were not made until 1941, when Martin and Synge reported on amino acid separation by liquid-liquid partition chromatography.⁶⁹ Following on, in 1952, James and Martin then developed the first gas-liquid chromatographic (GLC) column, when they described the separation of volatile fatty acids by partition chromatography with

nitrogen gas as the mobile phase and a stationary phase of silicone oil.⁷⁰ This paper is regarded by many as the first paper on GC and demonstrated that tiny amounts ($< 1\ \mu\text{g}$) of material could be separated by partitioning between the gas-liquid phases.

Gas chromatography uses a permanent gas such as nitrogen, hydrogen or helium as the mobile phase. Martin and colleagues discovered that by using a gas instead of a solvent for the mobile phase, the system was far more dynamic and more refined separations could be achieved. The use of thermal gradients can also improve the resolution in mixtures with volatile and semi-volatile components, by making use of their relative vapour pressures. Increasing the temperature influences the partition coefficient of the analytes between the stationary and mobile phase; when optimised, this can result in their focussing at the entry of the column and their elution into the detector in a narrow band, thus further enhancing compound resolution. Since the components of the mixture attain different migration rates, based on their interaction with the stationary phase, they will elute at different rates and under identical conditions will have highly reproducible retention times.

It was the development of the flame ionisation detector (FID), by McWilliam and Dewar, in the late 1950s that allowed the potential of GC to be fully explored.⁷¹ Unlike previous detectors, the FID was capable of very low background noise and as a result it was capable of complex separations at very low sample volumes ($< 1\ \mu\text{L}$) and had very fast response times ($\sim 1\ \text{ms}$).⁷² Pauling was the first to illustrate its power for biological samples, by demonstrating the detection of over 250 volatile organic compounds (VOCs) in both breath and urine samples.⁷³

With an increasing number of detectors becoming available GC columns also developed, from packed open tubes containing silicone oil to packed columns containing, for example,

activated charcoal or silica gel. It was the development of capillary GC columns with their improved separation efficiencies resulting in greater peak capacity (number of compounds that can be resolved) and shorter run times that really established gas chromatography as the powerful analytical tool it is today.

Capillary columns, as the name suggests, have narrow bores (typically 0.1 to 0.5 mm i.d.) and are usually manufactured from fused silica and are externally coated in a polyimide resin; although, de-activated metal columns now allow an increase in the upper operating temperature and are able to be used beyond the confines of the laboratory. They tend to be coated internally with a polysiloxane layer, the chemical composition of which can be modified to provide the most appropriate stationary phase material for the compounds to be separated. The columns can be wound tightly on to cages that enable tens of metres of column (15 or 30 m being used more often) to be housed in a standard GC oven. In addition, the analysis of highly volatile compounds are enhanced by the use of porous layer open tube (PLOT) columns instead of polysiloxane as the stationary phase. An additional consequence of the use of long, narrow tubes is that the peak capacity (separation efficiency) can be maintained with much lower volumetric flows (1-2 mL/min) of carrier gas than used in packed column systems (~20-40 mL/min), enabling the coupling of a GC to analytical detectors that require a vacuum for operation. It is the coupling of capillary columns with mass spectrometry that has fundamentally transformed the analysis and identification of components in complex matrices.

1.2.2. Mass spectrometry coupled to gas chromatography

The coupling of a mass spectrometer to a gas chromatography column provides the analyst with one of the most useful tools. Having resolved the components of a complex sample on

the GC column, the mass spectrometer enables the differentiation of molecules of different relative molecular mass. The standard measurement unit is the Dalton (Da), based on the ^{12}C isotope value of 12.0000.

The invention of mass spectroscopy is often attributed to J. J. Thomson, who studied electrical discharges in gases.⁷⁴ Thomson began working at the Cavendish Laboratory in Cambridge in 1880, and by using vacuum systems he was first able to show deflection of particles by magnetic and electric fields. Using this deflection he was then able to calculate the velocity of the particle. Using different gases, he obtained the same e/m value of the particles. He found that these were one thousandth of the mass of hydrogen. Thomson called the particles ‘corpuscles’ but were later to become known as electrons, a phrase coined by an Irishman, G. Johnstone Stoney.⁷⁵ Thomson made the first announcement of the existence of the corpuscles at the Royal Institution on the 30th of April 1897.^{76,77}

In 1907, Thompson began investigating positive rays hoping to identify particles that carried a positive charge. Ions of neon, generated in gas discharge tubes, were passed through parallel electric and magnetic fields in a parabolic trajectory; a photographic plate was placed in its path to measure the deflection. Thompson observed two patches of light on the photographic plate which he initially believed to be from another species but later recognised them as the two isotopes, neon-20 and neon-22. The mass of naturally occurring neon is 20.2, as the heavier isotope is present at ~10 % of the lighter isotope. The weak beam signal for the less abundant heavier isotope meant many were not convinced by Thompson’s findings.⁷⁸

Francis Aston joined Thompson at the Cavendish Laboratory in 1909 and in 1919 would go on to construct what has been recognised as the first mass spectrograph, the forerunner

of the mass spectrometer, for the separation of charged atoms based on their masses. The primary modification was that Aston allowed his particles to be deflected by the electric field before entering the magnetic field and the direction of the field was different. Ultimately it resulted in more accurate results and clearer photographs.⁷⁸

1.2.2.1. Electron ionization

Electron Ionisation (EI), the primary mechanism used in most modern mass spectrometers, was introduced by Dempster in 1921.⁷⁹ EI is a harsh ionization technique resulting in substantial molecular fragmentation. Despite this, the ionization process is highly reproducible, which can simplify compound identification and the fragmentation patterns generated can be used for compound fingerprinting.

The EI process itself requires the analyte to be in the gas phase. As shown in Figure 1.11, the electrons are emitted by the heated filament which are then focused into a region for interaction with the analyte molecules. Analyte molecules are introduced into the ionisation source and are ionised by the direct impact of a 70 eV electron, resulting in removal of an electron from the analyte molecules and the formation of positively charged radical cations. The ions formed are moved out of the ion source by an electric field. Depending on the lifetime of the excited state, fragmentation will either take place in the ion source, giving rise to stable fragment ions, or on the journey to the detector, forming metastable ions.⁸⁰

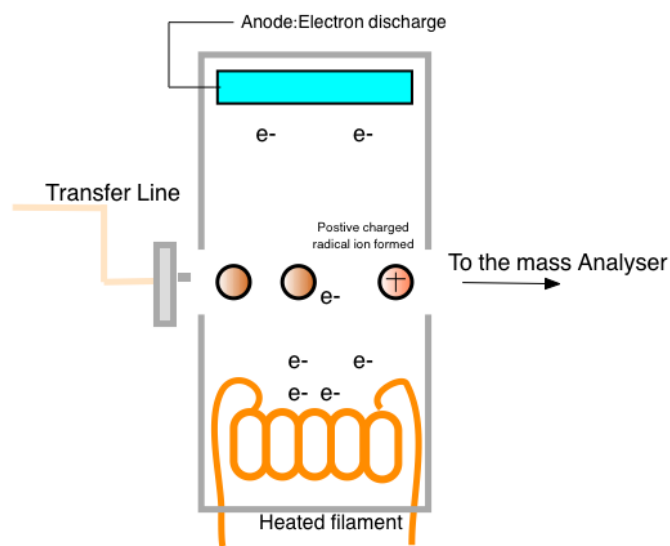


Figure 1.11. Showing the electron ionization (EI) process in mass spectrometers, with the eluent of the GC column entering via the transfer line and the charged radical ions exiting in to the mass analyser, prior to separation and detection.

The energy required to remove an electron from a molecule is termed the ionization energy (IE). This energy differs for each type of molecule. Most organic compounds are ionised in the IE range of 6 to 12 eV. At 70 eV, EI ensures an effective transmission of additional energy that induces a reproducible fragmentation of the molecule, which can enable structurally informative information to be derived from the mass spectra generated.⁸¹

The minimum energy required to form a fragment ion is called the appearance energy (AE) of this ion. The formation of fragment ions from the radical cations is caused by the excess internal energy present in the range of 0 to 20 eV. This high internal energy induces fragmentation of the molecular ion causing the molecular ion to fragment by breakage of chemical bonds. The bonds that are broken during the fragmentation process form a more stable ion.⁸¹ The distribution of energy among the ions formed, and the ensuing pattern of fragmentation produced from distinct chemical structures, depends on chemical bond strength and fragment ion stability. The EI ionisation process forms a basis for qualitative

and quantitative mass spectra analysis and interpretation. Furthermore, the extensive fragmentation can provide useful information into characteristic related to the structural features of a molecule under investigation.

EI combined with a full mass spectrum scan can provide the user with openings into interpreting a contaminated mass spectrum. Similarly, it can provide relevant information on ions that may allow the user to separate two unresolved chromatographic peaks. The mass spectrum of unresolved compounds or shoulder peaks can then be analysed for artifacts or suspected peaks hidden beneath a main peak. This plays an important role in the pharmaceutical industry, with the identification and quantification thresholds for impurity peaks set by the regulatory body. Peaks above the threshold require the identity of the impurity for toxicological studies to ensure no harm to patient and thus allow the product to be marketed.

Large spectral libraries of mass spectra (*e.g.* Wiley/NIST, 2008), consisting of full scan data obtained using electron ionization spectra are easily accessible. The collation of bespoke, in-house, compound specific libraries that are readily searchable for matching spectra can also be used for compound identification. However, it should be noted that a lack of a spectral match or a spectral mis-match can occur in trace analyses, usually caused by matrix interference or contaminated samples, and requires a skilled analyst to interpret the data.⁸¹

1.2.3. Comprehensive gas chromatography/mass spectrometry (GCxGC/TOFMS)

Comprehensive (two-dimensional) gas chromatography (GCxGC) was first described, in 1991, by Phillips and Liu.⁸² In comprehensive chromatography, two capillary columns with different stationary phases are connected by a modulator, in order to achieve orthogonal

separation. By employing two different stationary phases, and the modulator, all of the sample introduced on the first column also undergoes a separate separation in the second column, resulting in an increase in chromatographic resolution (how well peaks are separated) and peak capacity (number of peaks that can be separated on a column). Effectively, in comprehensive chromatography the peak capacities of the two columns are multiplied, hence the use of the term GCxGC. Through the above processes, comprehensive chromatography generates peaks that are much sharper and narrower (0.1-0.3 s) than in conventional one-dimensional gas chromatography (2-3 s). This not only increases their ability to be resolved from other analytes, matrix compounds and column bleed but also results in an increase in their signal/noise ratio, thereby allowing lower detection levels than in one-dimensional chromatography.

The nature of the peaks eluting from the second column in comprehensive chromatography imposes constraints on the detectors that can be used. Quantification of a gas chromatography peak necessitates ~ 20 data points to be collected, so that its peak shape is well characterized. When peaks are 0.1 - 0.3 s wide, this necessitates spectra acquisition rates of 50-100 Hz. A schematic of the primary components used in the commercial comprehensive chromatography system utilized in this study, is provided in Figure 1.12. The detector used is a time-of-flight mass spectrometer with unit-mass resolution in the range of 5- 1000 Da and an upper spectra acquisition rate of 250 Hz.

The Pegasus IV, from the Leco Corporation (St Joseph, Michigan), uses a standard Agilent 6890 GC into which a twin-stage thermal modulator (Leco Quad-Jet) and a secondary oven (both described below) have been added. A liquid nitrogen cryostat is also added and connected to the thermal modulator, via a valve, to allow cryogenic trapping of the primary column effluent on demand. An air supply and two heated jets make up the remainder of

the thermal modulator. The GC is fitted with a standard split/splitless injector for sample introduction.

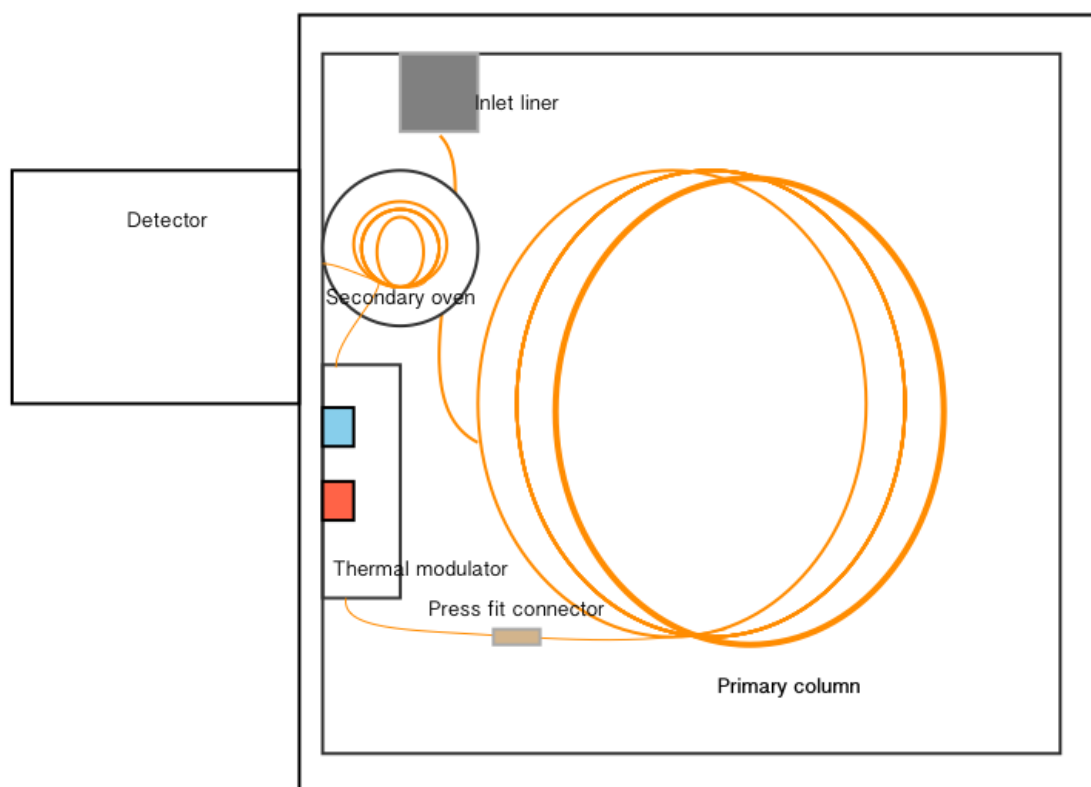


Figure 1.12. Showing the configuration of the Leco Pegasus IV, comprehensive gas chromatography system.

Optimization of the injection bandwidth of the eluent on to the second column, from the first, is key for the success of comprehensive chromatography and is achieved by a two-stage thermal modulator. The modulator consists of two cryotrap and two heaters that trap, focus and then deliver the analyte into the second column. The effluent from the first column is trapped and focused into a narrow band in the first stage, using a cold jet. The hot jet releases this narrow band into the cold 2nd stage. The 2nd stage then holds the sample as the 1st stage cools and traps the next band. Once the 1st stage is cold, the 2nd stage releases the sample into the secondary column. The 2nd stage then cools and the 1st stage releases the

next trapped sample. This cycle repeats continuously sampling slices from the first dimension. The first dimension peak is effectively split into several modulated peaks and then combined with the second dimension coordinates by the Leco ChromaToF software into a surface contour plot.

Conventionally, in comprehensive chromatography the analytes are separated on a non-polar column (primarily by volatility) in the first dimension, followed by separation on a more polar column in the second dimension. However, any combination of the vast array of commercial stationary phases are possible, as long as they are within the operating parameters of the system. The dimensions of the first column are as used in standard GC/MS applications (*i.e.* 25- 30 m in length, ~0.25 mm *i.d.*); whereas, the second column is much shorter (~1-2 m) and has a very small internal diameter (~0.1 mm). The size of the second column is driven by the requirement that the analytes have to elute from this column within the period of modulation (~4-10 s) applied to the first column. This modulation period is short and ensures that any separation achieved in the first dimension is preserved.

Since this study started, Zoex (UK) Ltd now distribute an alternative thermal modulator and 2D software, which has been developed by its parent US company. Their modulator utilizes a sampling loop and only one hot and cold jet. It is reported to reduce peak tailing in the second dimension, further enhancing compound resolution and quantification.

1.2.3.1. Time-of-flight mass spectrometer

The time-of-flight mass analyser was originally described by Wiley and McLaren in 1955.⁸³ In contrast to other mass spectrometer systems in regular use in analytical laboratories, such as a quadrupole or ion trap, a time-of-flight mass spectrometer (TOFMS) does not require electronic and radio frequency fields to be scanned to cover the mass range

of interest; making it ideal for applications such as comprehensive chromatography where very high sample acquisition rates are required.

A time-of-flight mass analyser, as shown in Figure 1.13, measures the time it takes an ion to travel a fixed distance in a field-free region. The basic principle is that ions of different masses having the same kinetic energy will reach the detector at different times. Heavier ions will take longer to reach the detector than lighter ions. If the above underlying principle is to hold true then the initial kinetic energy of the ions entering the flight tube must be the same, otherwise the resolution ($\Delta m/m$) of the mass spectrometer will be compromised.

Ions formed by the EI process are pulse accelerated, as a packet of ions, from the ion source into the field-free region (flight tube) for separation. The expectation is that the ions have almost the same kinetic energy and so their velocities will depend only on their mass to charge ratio (m/z). However, the actual position of their generation within the ionisation source will differ and so ions will be generated with a spread in kinetic energy.

In order to reduce the influence of the spread in kinetic energy on the times-of-flight of the ions and to improve the instrument mass resolution, an electrostatic device called a reflectron is placed in the drift region. A reflectron is a series of electrostatic lenses that reduces the overall length of the mass spectrometer and is maintained at a particular distance from the ion source and the detector. Ions having higher kinetic energy penetrate deeper into the reflectron than lighter ions and thus travel further. Effectively the ions are packeted, thus reducing the flight time distribution and resulting in an increase in resolution.

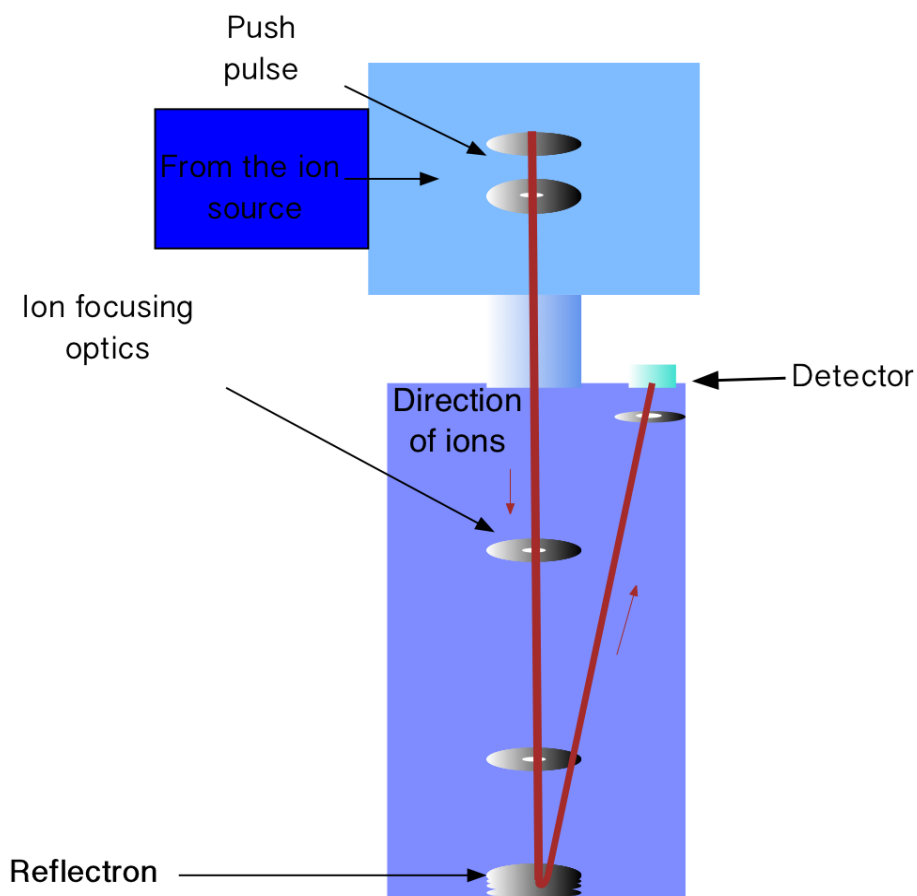


Figure 1.13. Showing the basic elements of a time-of-flight mass spectrometer: the ionisation source with its push pulse plate; the ion focussing optics and reflectron in the flight tube, and the secondary electron multiplier (SEM).

1.2.3.2. Applications of comprehensive chromatography

Comprehensive two-dimensional gas chromatography (GCxGC) has increasingly been recognized as a technique capable of providing improved resolution of compounds within complex matrices when compared to conventional, single dimension GC.^{84,85} Amongst others, the technique has been successfully applied to petrochemical, environmental and food analysis.⁸⁶ Kueh *et al.*, first described the application of comprehensive GCxGC for anti-doping control in equine and canine sports.⁸⁷ Song *et al.*, went to apply it to a drug standard mixture containing over 78 drug compounds.⁸⁸ In our own laboratory, Bill

Guthery has explored the qualitative determination of various opiates and benzodiazepines in human serum and the qualitative detection of various drug types, including opiates, cocaine, methadone and diazepam, in hair samples using GCxGC/TOFMS.^{89,90,91}

During the period of this study, comprehensive gas chromatography has separately been applied in the field of anabolic steroids.^{92,93,94,95,96} The listed publications share our hypothesis that comprehensive gas chromatography can be relevant to steroid detection. Its application as a screening process, the detection of designer steroids and its coupling with molecular imprinted polymers has not, to the best of my knowledge, been published.

1.2.4. Mass spectrometry coupled to liquid chromatography

The development of high performance liquid chromatography (HPLC) has reduced the need for complex clean-up procedures and derivatisation and when coupled to mass spectrometry has replaced gas chromatography/mass spectrometry as the preferred analytical technique for certain target species.⁹⁷

A triple quadrupole mass spectrometer enhances the capabilities of a traditional scanning quadrupole mass spectrometer, by allowing the user to study mass transitions (MS/MS). A triple quadrupole MS actually consists of two quadrupoles and a collision cell which enables the molecular ion (parent ion) to be isolated by the first stage of the instrument and then fragment (daughter) ions to be generated through the application of a collision energy. These fragment ions are then separated in the secondary quadrupole region prior to detection. The process is known as selected reaction monitoring (SRM), and is an extension of selected ion monitoring (SIM) in traditional quadrupole and ion trap mass spectrometers. These highly diagnostic transition spectra give greater proof of the target ion and provide a higher sensitivity with modern triple quadrupoles detecting < 50 pg at the point of analysis.

When a triple quadrupole mass spectrometer is coupled with a liquid chromatography system a different ionisation process is required to the standard electron impact (EI) source used in gas chromatography/mass spectrometry.

1.2.4.1. Electrospray ionisation

The process of ion formation in electrospray ionisation (ESI) is complex and requires high voltage. Despite this ESI is often referred to as a “soft” ionization technique and this relates to the fact that the analyte’s structure remains intact, with very little fragmentation of the molecule occurring during the transfer of the ion from solution into the gas phase. This unique aspect of electrospray ionisation (ESI) has been revolutionary in the field of biological mass spectrometry, increasing the range of compounds analysed to include large, thermally labile and non-volatile compounds such as nucleic acids and proteins. Mass analyzers measure the mass to charge (m/z) ratio of molecules and have a specific range. Often biological molecules are high molecular weight compounds and are beyond the mass range possible for analysis. ESI allows multiple charge formation. The advantage is that the overall charge of the molecule increases whilst at the same time reducing the m/z values of the ions formed, thus enabling the analysis of molecules of high polarity and molecular weight.⁹⁸

Electrospray was first introduced by Dole *et al.*, in 1968, as a method to charge synthetic polymers.⁹⁹ The pre-requisite of the technique is that the analyte be: a) ionized and b) in solution. The solution is pumped through a metal capillary, held at high voltage, by an HPLC pump. The transformation of analytes into gas-phase ions occurs at atmospheric pressure. The analyte is first dissolved in a volatile solvent and forms ions by the addition or removal of a hydrogen ion. The addition of hydrogen produces the positively charged

molecule ion, $[M+H]^+$, whilst negatively charged ions are produced by the removal of a hydrogen ion, $[M-H]^-$. The ions formed migrate to the tip of capillary needle held at high voltage, ~ 3 kV. This electric field induces a charge accumulation at the liquid surface. With increased repulsion at the tip, caused by ions accumulating, the liquid forms a Taylor cone. At a crucial interval, and due to increased Coulombic repulsive forces, the liquid breaks away from the tip of the Taylor cone to form highly charged droplets. The droplets' release is aided by de-solvation by the nebulising gas (nitrogen) flowing outside of the capillary. Smaller droplets with an increasing charge-to-surface area ratio form, and gas phase ions are ultimately produced from the progressively smaller droplets.

The formation of gas phase ions from these highly charged droplets is thought to proceed through two mechanisms, the ion evaporation model (IEM) and the charge residual model (CRM).¹⁰⁰ Solvent evaporates from the droplets, leading to a decrease in droplet radius but no change in charge. At a particular radius, for a given charge, the charge repulsion (or Coulombic repulsion) within a droplet exceeds the surface tension, which results in Coulombic fission. The point at which this occurs is known as the Rayleigh Limit. The IEM proposes that these solvent evaporation and Coulombic fission cycles continue until droplets are of a certain radius, at which direct emission of ions into the gas phase occurs. The CRM model suggests that these evaporation/fission cycles continue until there is no more solvent remaining and only the charged analyte remains. There is a general consensus that ions with a small number of charges, at low m/z values, are preferentially formed by the IEM whilst multi-charged ions, at high m/z values, are formed by the CRM.¹⁰¹

1.2.5. Molecular imprinted polymers (MIPs)

1.2.5.1. Limitations of existing extraction methods

As reported in the section on nutritional supplements, the detection of trace amounts of anabolic androgenic steroids, their metabolites and prohormones in nutritional supplements is highly challenging. Despite legislation, the range of compounds available to athletes is ever increasing, as are the ways of trying to conceal or mask the presence of a prohibited compound. Some of the issues in the screening of these substances are a result of the inherent limitations in the detection methods used; however the sample preparation method also contributes to the issue.

As summarised in Section 1.1.2.2, an extensive clean-up procedure is required for the analysis of steroids in urine to enable their detection at the levels required by WADA. This is usually achieved through the use of solid phase extraction (SPE) cartridges. A wide range of sorbents can be used for conventional solid phase extraction, which include C8 and C18 bonded phases on silica, polymeric resins, polar sorbents such as alumina, charcoal, silica and cyano & amino-bonded sorbents.

Conventional SPE sorbents retain targeted analytes by non-selective means, such as hydrophobic interactions that can result in co-extraction of interfering matrix species. Urine samples are inevitably highly variable, the levels of matrix components such as amino acid, urea and water content can vary significantly. Similarly, compounds such as bilirubin may also affect the extraction process and reproducibility for the sample analysed. Variations in the sample matrix can also negatively affect the ability of the sorbent to extract the analytes of interest. The limitations of existing extraction methods has prompted the exploration, in this thesis, of a robust, more selective extraction process for the

screening of a range of steroid molecules, this alternative extraction process is known as molecular imprinted polymers (MIPs).

1.2.5.2. A brief history of MIPs

Whether it be enzymes, antibodies or receptors, molecular recognition and interaction is vital for biological function.¹⁰² Any disturbance in the human body's mechanism to recognize and bind to the specific molecule can lead to malfunction and disease. This 'lock and key' model for interactions, initially proposed by Fischer to explain enzyme-substrate recognition, has been utilised by chemists for the development of synthetic receptors, called molecular imprinted polymers.^{103,104,105}

Molecular imprinting is based on the synthesis of a highly cross-linked polymer, built around a template (representative of the target), in the presence of a suitable functional monomer and cross-linker. As shown in Figure 1.14, a pre-polymerisable complex is formed between the template molecule and one, or more, functional monomers. Polymerisation is then initiated, either with heat or by photolysis, in the presence of an excess of cross-linking agent to produce the MIP. By removing the template molecule, numerous cavities remain with a well-defined size and shape and the desired functional groups which can act as selective recognition sites, *i.e.* artificial receptors, for molecules of interest.¹⁰⁵ Target molecules can then be "recognised" by the polymer through size exclusion and the potential formation of hydrogen bonds, ionic interactions, hydrophobic binding, formation of reversible covalent bonds, *etc.*¹⁰⁴

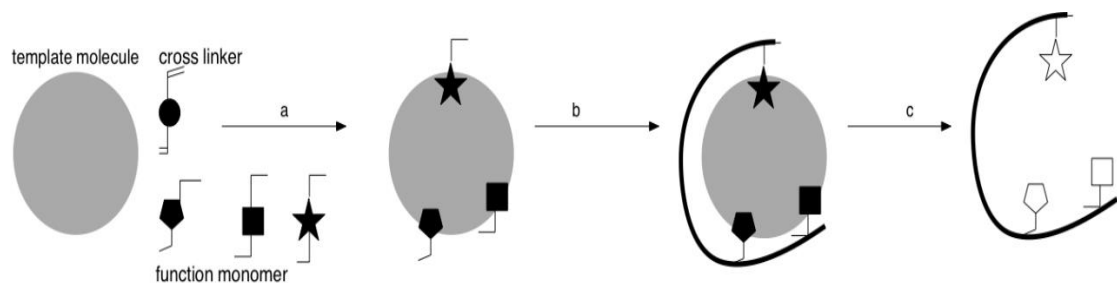


Figure 1.14. Showing how MIPs are generated (a) functional monomers and cross-linker molecules are mixed together forming a complex with the template molecule, (b) the functional monomers co-polymerizes with the cross-linker forming a highly cross-linked polymeric network, (c) removing the template releases complementary binding sites that can rebind the template.

In the 1930s, a Ukrainian group led by Polyakov developed the first synthetic materials with biomimetic recognition properties.^{106,107,108} They showed that silica gel prepared in the presence of organic solvents had an affinity towards the template molecule. Their work was not widely recognised until 1947, when Linus Pauling's student, Francis Dickey, developed silica gels for methyl orange and its derivatives. The "footprint" theory proposed by these authors was eagerly accepted and actively pursued by other researchers.¹⁰⁴

Since a revival of interest in the subject in the 1970s, chemists have synthesized a wide range of molecular imprinted polymers that combine the advantages of synthetic plastics, such as low cost, durability and robustness, with the recognition properties of natural receptors. In 1972, Wulff and co-workers published the first formal methodology for molecular imprinting in organic polymers.¹⁰⁹ Many companies now sell tailor-made MIPs, for example MIP Technologies AB; POLYIntell and Semorex Inc.¹⁰⁵

The preparation of an optimised molecular imprinted polymer depends on the selection of the most appropriate combination of the constituent parts for the particular application and target species of interest. Their selection can be achieved through theoretical modelling of the system, or more likely through empirical evaluation based on practical experience of

what MIP recipes have been used in the past. Further information on the primary components that are utilised to develop MIPs are summarised below.

1.2.5.3. Functional monomer

The choice of the functional monomer plays an important role in monomer-template complexes during the pre-polymerisation imprinting process. The functional monomer is chosen to assist the formation of strong interactions between itself and the template. The non-covalent imprinting approach is the most frequently used in imprinting and was developed by Mosbach and Arshady in the early 1980s.¹¹⁰ It has the advantage of being simpler to implement than covalent imprinting, is open to a wider range of templates and generates a greater number of high affinity sights.^{104,105} Acidic, basic and neutral monomers are regularly used for MIPs generation. Some frequently used non-covalent functional monomers (acidic and basic) are shown in Figure 1.15. The most common method for forming imprinted polymers is free radical vinyl polymerisation involving non-covalent interactions.¹⁰⁵ The most frequently used functional monomers are methacrylic acid (acidic) and 4-vinylpyridine (basic).

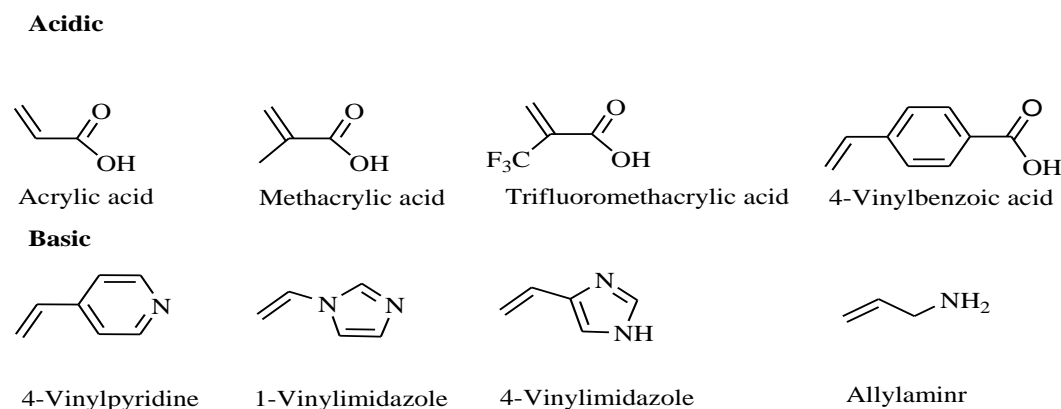


Figure 1.15. Representative functional monomers (acidic and basic) used in the preparation of molecular imprinted polymers (MIPs).^{104,105}

Whilst non-covalent imprinting has significant advantages over covalent bonding imprinting, the larger excess of monomers used can result in more non-specific binding sites, which can result in lower polymer specificity. The binding and release of template is efficient due to the weak electrostatic (e.g. ionic, dipole-dipole interactions), hydrogen bonding and Van der Waals interactions between the polymer and template and can be affected using mild changes in environment. As a result the polymerisation conditions need to be carefully chosen to maximise the polymer template interactions and preserve the formed complexes during the polymerisation stage.¹¹¹

1.2.5.4. Initiator

The polymerisation mechanism involves three distinct phases: initiation, propagation and termination. During initiation, radicals are formed by thermal or photochemical decomposition of the initiator, producing a metastable radical which reacts with the vinyl monomer to form a heterodimer, which contains a free radical. The polymerisation reactions then continues, and propagation occurs rapidly by the addition of a new monomer thus growing the radical chain. Termination then occurs either by the removal of the thermal source, dimerization of the radical chains or exhaustion of the monomers. Polymerisation should be performed in an inert atmosphere, usually nitrogen, to prevent chain termination through reaction of the radical with oxygen. A typical radical initiator, 2,2-azobisisobutyronitrile (AIBN) is shown in Figure 1.16.

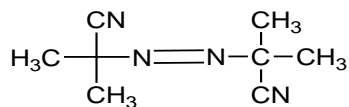


Figure 1.16. 2,2-azobisisobutyronitrile (AIBN), a typical radical initiator used in the preparation of molecular imprinted polymers (MIPs).^{102,103}

1.2.5.5. Cross-linking agent

The interaction of the monomer and the template and then subsequent removal of the template results in the formation of functional residues. Uniform distribution of these functional groups ensures an efficient imprinting and re-binding process for the target species. In order to achieve this uniform distribution within the polymer, a cross-linking agent is required. Common cross-linking agents are shown in Figure 1.17.

Cross-linking plays an important role in preserving the binding specificity of an imprinted polymer. When functional groups are not sufficiently constrained by cross-linking, the binding specificity decreases. Conversely, an overly rigid structure formed by too much cross-linking may result in a decrease in loading capacity, caused by a restricted access of the analyte to the binding regions.

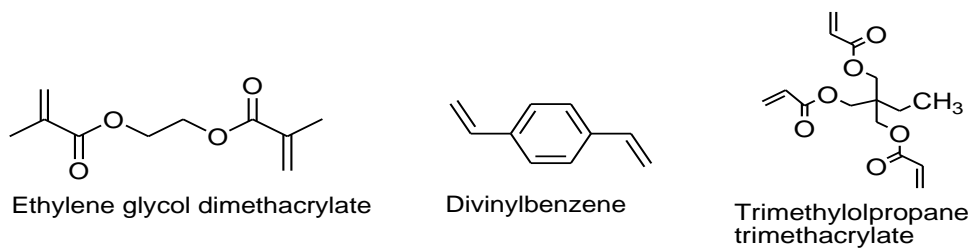


Figure 1.17. Representative cross-linking agents used in the preparation of molecular imprinted polymers (MIPs).^{104,105}

As can be seen in Figure 1.17, trimethylolpropane trimethacrylate (TRIM) has one more vinyl group than ethylene glycol dimethylacrylate (EGDMA) and therefore has the potential to generate greater three-dimensional backbone rigidity, contributing to a higher binding specificity.

1.2.5.6. Solvent

Several groups have reported that the physical state (toughness, ability to swell, morphology, pore size distribution and structure) of the MIPs and the imprinting process itself is dictated by the choice and the amount of the porogenic solvent used in the polymerisation recipe.¹⁰⁵ The solvent selected will solubilise all the monomers in the pre-polymerisation mixture, it will also stabilise the template-monomer complex and will help control the porosity of the resulting MIP. In non-covalent imprinting a non-polar solvent is preferred over a polar one, which would interfere with the electrostatic interactions needed for a successful imprint; for example, toluene and chloroform are widely used in imprinting.

1.2.5.7. MIPs for the selective extraction of steroids

The use of MIPs for the extraction and clean-up of steroids has not, to the best of the author's knowledge, previously been reported; however, Shi *et al.*, have reported selectively extracting cholesterol from different matrices including human serum, cow milk, yolk, shrimp, pork and beef samples using a MIP.¹¹² Their results showed high affinity and binding of their MIP for cholesterol. According to the authors, the matrix interferences were almost completely removed after molecular imprinted solid phase extraction (MISPE). The authors also emphasised that the yolk extracts, following MISPE, had better baselines, higher recovery and selectivity than that obtained after C18 solid phase extraction (SPE).¹¹²

In this thesis, the aim was to develop non-covalently bonded molecular imprinted polymers that were capable of selectively extracting a range of steroids from urine. Testosterone was used as the template molecule with the view that once it was removed the cavities

generated would be complementary in shape, size, affinity and functionality and would therefore bind to a wide range of endogenous androgens, 19-nor-steroids, metabolites, prohormones and synthetic analogues of testosterone (designer steroids) that share the same four ring, fused nucleus as testosterone. Following elution of these steroids from the MIPs they will be analysed by comprehensive gas chromatography to provide a screening method for steroids present in complex matrices.

Although the method will be used for the extraction of steroids from spiked synthetic urine it is anticipated that in the future it may also be applied to different biological and food samples such as human serum, contaminated meat and nutritional supplement with slight modification to accommodate solid sample analysis.

1.2.5.8. The application of MIPs with steroids in the thesis

As has previously been stated in Section 1.1.2.2, it is recognised that most steroids present in urine samples are actually present in the conjugated form, as a result of Phase II metabolism, to form either a sulphate or glucuronide moiety. It is also recognised that less than 3% of the total androgens excreted via urine are unconjugated, *i.e.* in the free form. Conjugation of steroid androgens is generally with glucuronide; however, steroid androgens such as testosterone, epitestosterone, androsterone and etiocholanolone may also be excreted as sulfates, with their ratio to glucuronide nearing 1:1, in some instance.

As both sulfates and glucuronide conjugates are formed through metabolism, they are beyond the scope of this study, due to the significant practical and ethical approval constraints that this would have introduced in the project. In addition, none of the conjugates would be viable for comprehensive gas chromatography, as they are not volatile nor thermally stable.

The approach taken in this project, to evaluate the performance of the MIPs with synthetic urine spiked with steroids, is based on the fact that free steroids are available in urine and that many existing methods already utilise deconjugation or hydrolysis of the steroid conjugate prior to solid phase extraction, followed by derivatisation ahead of gas chromatographic separation. In addition, prohibited AASs in nutritional supplements, once extracted with a suitable solvent, will be in the free form and positive control samples for supplement testing are prepared by spiking with the steroid. The coupling of a free steroid MIPs method to the steroid deconjugation process and extraction methods used for nutritional supplement is beyond the scope of this thesis and should be addressed in the future, once the performance has been evaluated.

1.2.6. Aptamer-based detection

In addition to molecular imprinted polymers, antibodies have been a popular choice for molecular recognition in diagnostic applications for more than four decades and have made an enormous contribution to a wide range of diagnostic assays routinely used in clinics.¹¹³

Despite their widespread application, antibodies are not without their limitations:

- i) the production of monoclonal antibodies is a labour intensive and expensive process,
- ii) the generation of antibodies for toxins remains difficult, because the process begins in an animal,
- iii) the antibody may show variation from batch to batch, thus affecting the binding affinity,
- iv) an antibody can be irreversibly denatured by high temperatures.

Given these factors, aptamers have emerged, since the early 1990s, as an alternative to antibodies and molecular imprinted polymers in both diagnostic and therapeutic applications.^{113,114}

Aptamers (from the Greek words meaning “fitting” and “particle”) are three-dimensional structures with a well-defined single stranded sequence of oligonucleotide entities. They tend to be short (50-100 bases) and can be composed of RNA, DNA or modified bases. A large number of aptamers can be generated randomly in an oligonucleotide library and then those ligands that bind *in vitro* to a given target solute, with high affinity and selectivity, can be amplified for use in applications.¹¹⁵ The *in vitro* selection method is known as SELEX and has been widely developed, from that first reported by Gold's and Szostak's groups in 1990.^{114, 116, 117}

In brief, SELEX uses a nucleic library (10^{14} - 10^{15} random oligonucleotide strands) which is incubated with the target molecule. The bound and unbound strands are separated and the target-bound strands are eluted from the target molecule and are amplified via a polymerase chain reaction. This selection process is repeated between 6 and 15 times, with increasingly stringent conditions, so that the nucleic acid obtained has the highest affinity to the target molecule.¹¹⁴

The interest in current research remains based on their high affinity, specificity and reversible denaturation. The range of molecules that aptamers can bind to include nucleic acids, proteins and small organic molecules.¹¹⁸ Aptamers have been designed for targets that cover a wide range of diseases from HIV to tropical diseases and cancer.^{118,119} The versatility that aptamers display for target recognition have made them a good candidate for analytical diagnostic applications.

Aptamers overcome a number of the issues associated with the use of antibodies:

- i) their production does not require the use of animals and therefore they can be produced against a number of toxins,
- ii) as they are produced by chemical synthesis, with high accuracy and reproducibly, they show little or no batch-to-batch variation,
- iii) aptamers can undergo modification to enhance their binding affinity to target molecules, which is a major advantage compared to antibodies,
- iv) aptamers elicit little or no immunogenicity in their therapeutic application.

1.2.6.1. Attaching aptamers to magnetic beads

In 1976, Prof. Ugelstad and co-workers were successful in producing polystyrene beads that were spherical and highly reproducible in size, a feat previously only achieved in zero-gravity.¹²⁰ The group then succeeded in making these beads magnetisable and they were commercialised by Invitrogen.

Dynabeads M-280 (Streptavidin), from Invitrogen (Life Technologies), are used for the attachment of biotinylated molecules. Having a monolayer of recombinant streptavidin covalently coupled to the surface, these uniform and superparamagnetic beads are 2.8 μm in diameter. Most published research literature utilises aptamers bound via streptavidin-biotin.

Magnetic beads not only ensure an optimal large surface area to immobilise the analytes on, but also provide an easy mechanism by which the target-bound beads can be removed from the supernatant. Traditional sample-enrichment processes, such as solid phase extraction, can require several complicated procedures and skilled analysts.

Aptamers immobilised on magnetic beads have already provided a solution to the high throughput screening demands of the sexually transmitted disease, chlamydia. At the time of analysis, an aptamer for testosterone was not commercially available. Therefore aptamers were considered that bound to estradiol, as the compound most closely relate to our steroids of interest.

The goal of this work was to design and evaluate, through a short pilot study, the feasibility of an aptamer-based assay for the extraction of estradiol from complex matrices, prior to quantitation by mass spectrometry.

Despite recent advances, the use of GC/MS coupled to an aptamer-based enrichment procedure remains elusive. To the best of my knowledge there is no method in literature using comprehensive gas chromatography/mass spectrometry with aptamer coated magnetic beads for such sample enrichment.

1.3. Thesis overview

The aim of the thesis is to develop and evaluate the potential of a non-targeted analytical screening technique that enables the extraction and detection of as wide a range of steroids as possible, from complex matrices, at levels that are appropriate for the challenges of sports and supplement testing and clinical applications.

The analytical method to be investigated is comprehensive gas chromatography/time-of-flight mass spectrometry coupled with molecular imprinted polymers. The hypothesis is:

- i) by harnessing the orthogonal separating power of two-dimensional gas chromatography it will enable very similar compounds to be resolved temporally,

enabling its application for the screening of a wide range of derivatised and underivatised steroids.

- ii) the coupling of the comprehensive chromatography eluent with the fast acquisition rate of a time-of-flight mass spectrometer further enhances the resolving power by enabling the capture of the full mass spectrum at such a rate that peak deconvolution is also possible.
- iii) molecular imprinted polymers can be templated with testosterone with the aim of selective extracting a wide range of steroids from complex matrices, such as urine. This approach focuses on the shape-matching of the common steran nucleus of steroids and the associated binding with ketone and hydroxyl polar groups.
- iv) in contrast to existing targeted techniques, such as LC/MS/MS or GC/MS (SIM), this non-targeted screening process will also aim to capture a full mass spectrum profile of any steroid present in the sample; thereby reducing the potential for false negatives and will provide a highly visual chromatographic landscape so that the operators can concentrate their search for doping violations in specific regions (*e.g.* stimulants, prohormones or designer steroids) which are distinct from those of matrix compounds and instrumental artifacts, such as column bleed,

The developed screening technique will be evaluated for its suitability for detecting a range of structurally similar, difficult to analyse, sports testing relevant prohormones and a smaller range of new designer steroids, such as THG, which share the same ring structure as testosterone. The quantities and concentrations of prohormones and designer steroids analysed in the thesis, both as standards and in spiked synthetic urine, have been dictated by the Minimum Required Performance Level (MRPL) specified by the sports testing regulatory agency, the World Anti-Doping Agency, in its Prohibited Lists.^{5,121} The

experimental work was conducted based on the MRPL concentration level of 10 ng/mL, for steroids in urine, as published in 2010. During this period of study, WADA have since reduced the MRPL levels, for most steroids to 5 ng/mL. Discussions of the results and the evaluation of the applicability of the method will take in to account both these values.

Prior to evaluating the performance of MIPs coupled with comprehensive gas chromatography, a pilot study was conducted that set out to evaluate the potential of utilising aptamers bound to magnetic beads as a simple, extraction clean-up procedure for estradiol, prior to analysis by gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS/MS). The results of this study are presented in Chapter 5.

All analyses were performed at the Department of Physical Sciences, (formerly Planetary and Space Sciences Research Institute (PSSRI)) in the Science Faculty, at The Open University. Access, training and support was available for comprehensive gas chromatography and a Thermo Fisher triple quadrupole LC/MS/MS system. Access to both instruments for steroid analysis provided an exceptional opportunity; however, whilst there was experience of applying comprehensive chromatography to drug testing, there was no tradition in steroid analysis and the work presented in this thesis has been developed within the timeframe of this project. Similarly, there was no tradition of steroid analysis in the aptamers and molecular imprinted polymers groups.

The foregoing chapters describe a journey from the initial challenges of steroid detection to a solution which combines the selectivity of a molecular imprinted polymer and the separation capabilities of comprehensive gas chromatography. This screening method will

then be applied to the detection of designer steroids modified specifically to avoid detection.

Chapter 2: Outlines the experimental methods, the chemicals and reagents, the instrumentation and the preparative procedures undertaken for this thesis. The extraction methods and instrument operating conditions have been developed by a mixture of experience, critical review of published literature, personal communications and a degree of trial and error.

Chapter 3: The resolving power of comprehensive two-dimensional gas chromatography (GC x GC) coupled with time-of-flight mass spectrometry (TOFMS) is evaluated for a range of challenging prohormones. The twelve prohormones, shown in Figure 1.18, were provided by HFL Sports Science, and were selected as they are known to cause them issues: such as poor quantitation, high detection limits, poor peak shapes, unreproducible derivatisation and laborious and time-consuming extraction techniques.

Both derivatised and underivatised prohormones will be explored. Their elution times, limit of detection and linearity will also be determined. The efficiency of two molecular imprinted polymers (templated with testosterone in this project) to recover these prohormones from spiked synthetic urine samples (10 ng/mL) will be characterised.

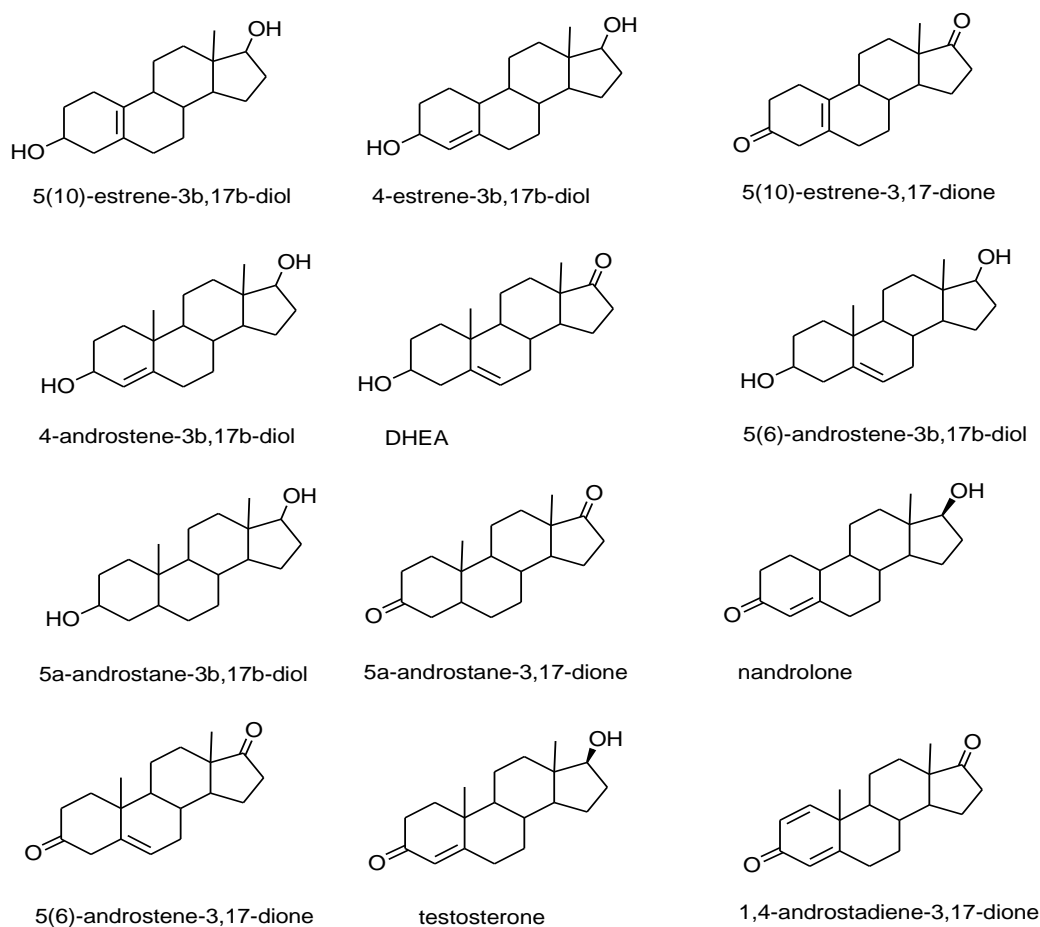


Figure 1.18. Prohormones to be investigated in this thesis.

Chapter 4: The method developed was applied essentially unmodified to screen for the presence of an additional six designer steroids to evaluate the wider, non-specific capability of the screening process. The designer steroids, shown in Figure 1.19, were provided by HFL Sports Science, as they are difficult to detect using the existing derivatisation methods employed in most sports testing laboratories. Those with functional groups added at the C-17 position are particularly difficult to derivatise, due to steric hindrance.

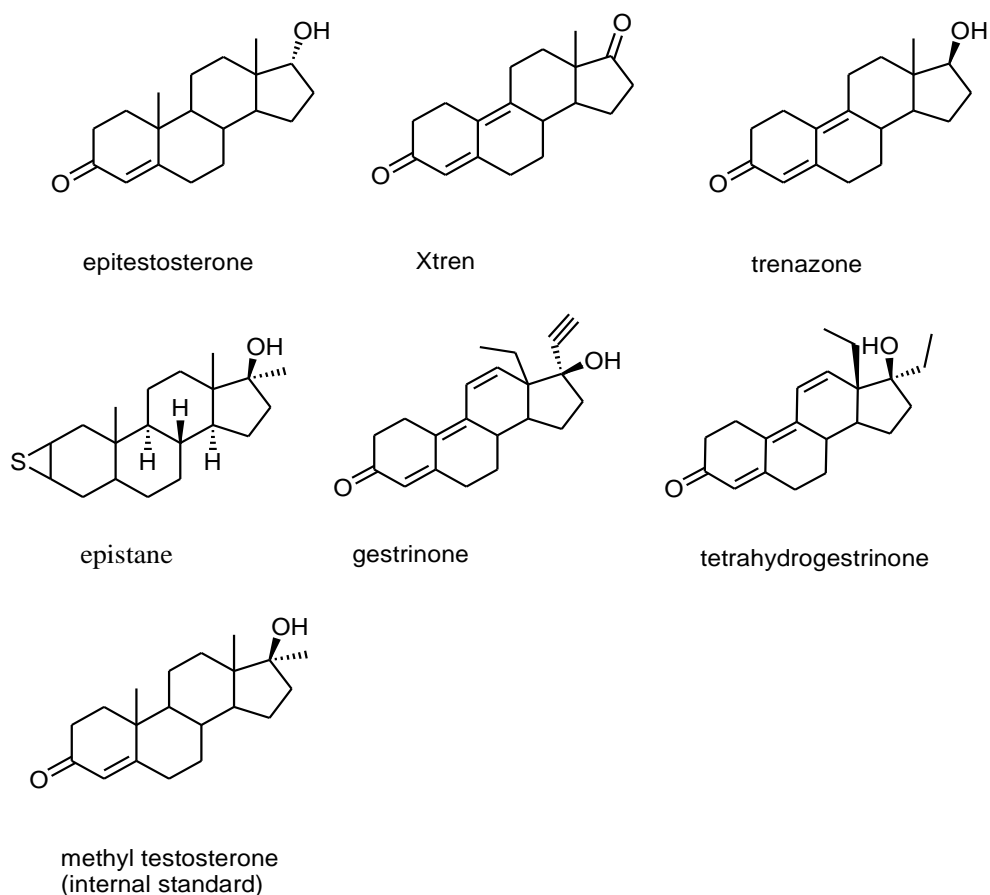


Figure 1.19. Designer steroids screened using the method developed in Chapter 4.

Prior to spiking into synthetic urine, a rigorous validation of the suitability of the comprehensive gas chromatography/mass spectrometry (GCxGC/MS) for the quantitation of prohormone and designer steroids in standard mixes was conducted, at levels appropriate to sports testing at the time of study. The performance of the molecular imprinted polymers was re-evaluated using designer steroids spiked into synthetic urine at the 10 ng/mL level (WADA 2010) along with the original cohort of prohormones spiked at between 2-10 ng/mL, to explore if they had any influence. Methyltestosterone was also used as an internal standard. To our knowledge, no comprehensive GC x GC screening method that utilises molecular imprinted polymers and detects designer steroids has yet been developed.

Chapter 5: In today's modern analytical laboratories, automated systems are constantly being pursued. The acceptance criteria for new instruments or techniques include their longevity, the expertise required to operate them and the ability to reduce cost. A critical measure of a laboratory's performance, investigating hundreds of urine or nutritional samples, is simply the number analysed in a day.

Mass spectrometry-based systems, when coupled with chromatography, can provide the ultimate specificity for compound identification and are therefore widely implemented. However, there is a desire to evolve from existing, mainly manual, solid phase extraction methods, due to the labour intensiveness and associated solvent cost.

In an attempt to bridge this gap, a pilot study was conducted to evaluate the hypothesis that small uniform magnetic particles coated in an aptamer (a complimentary molecular recognition technique to MIPs) could provide a simple sample clean-up method for the detection and quantification of estradiol by mass spectrometry. Accurate determination of the concentration of this endogenous hormone has a range of clinical applications.

Chapter 6: Conclusions and future work.

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2. Experimental

2.1. Introduction

This chapter is divided into two sections, based on the individual studies conducted within this thesis. The first section (Section 2.2) relates to the methodologies applied during the development and evaluation of molecular imprinted polymers for the selective extraction of prohormones from synthetic urine, followed by analysis by gas chromatography/mass spectrometry. The section is organised to provide a full description of materials and equipment used in each study and any preparative methods applied and instrumental settings used and is the basis of the studies reported and discussed in Chapter 3. It also describes the mixed steroid standards prepared for the more detailed validation study reported in Chapter 4 and the application of the method to a range of designer steroids. The method is then applied to evaluate the performance of MIPs with synthetic urine samples spiked with lower levels of prohormones (2 ng/mL) and designer steroids (10 ng/mL).

The second section (Section 2.3) describes the materials, apparatus and methods used as part of a pilot study to evaluate the detection and quantitation of estradiol following extraction by an aptamer bound to magnetic beads. The results of this study are reported and discussed in Chapter 5.

2.2. The development of a method for the analysis of prohormones and designer steroids by comprehensive gas chromatography/mass spectrometry (GCxGC/MS) following extraction by molecular imprinted polymers (MIPs)

2.2.1. Materials

The steroids used in this study were supplied by HFL Sport Science (Newmarket, Suffolk, UK), and form part of their screening process. Individual steroids were provided at

concentrations of 1 mg/mL and 10 µg/mL in methanol. The following 12 steroids were initially provided: 5(10)-estrene-3b,17b-diol, 4-estrene-3b,17b-diol, 5(10)-estrene-3,17-dione, 4-androstene-3b,17b-diol, DHEA, 5(6)-androstene-3b,17b-diol, 5a-androstane-3b,17b-diol, 5a-androstane-3,17-dione, nandrolone, 5(6)-androstene-3,17-dione; testosterone and 1,4-androstadiene-3,17-dione. All steroids were handled in accordance with Class C, Schedule 4 (Part II) Regulations. REA1090 - Buffer solution (pH 9.00) was purchased from LGC Ltd. (Teddington, Middlesex, UK). Surine (synthetic urine) was purchased from Cerilliant (Sigma-Aldrich Company Ltd., Poole, Dorset, UK).

All solvents used (acetonitrile, hexane, methanol, undecane) were of HPLC grade and were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Derivatising agents (ammonium iodide (NH₄I), Di-methyl formamide (DMF), N-methyl-(tert-butyldimethylsilyl)-N-trifluoroacetamide (MTBSTFA) and ethanethiol) were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK).

Methacrylic acid (MAA), 4-Vinylpyridine (4-VP), trimethylolpropane trimethacrylate (TRIM) and azo-*N,N'*-diisobutyronitrile (AIBN) were all obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK) for the preparation of the molecular imprinted polymers. All solvents used at this stage (acetic acid, acetone, acetonitrile, chloroform, methanol) were of HPLC grade and were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Testosterone was obtained through the Science Faculty Chemical Health and Safety Advisor (The Open University, Milton Keynes, UK) and used in accordance with Class C, Schedule 4 (Part II).

2.2.2. Apparatus and equipment

Vortexing was performed using the MS3 Basic (IKA, Staufen, Germany). Centrifugal evaporation was performed using a SF50 and CVP100 pump (Genevac Ltd., Ipswich, Suffolk, UK). Heating was performed in Techne DB-3A dry block heater (Bibby Scientific, Staffordshire, UK). Glass vials were obtained from Chromacol Ltd. (Welwyn Garden City, Herts, UK). Solid-phase extraction (SPE) and molecular imprinted polymer extraction (MIP) were performed using a 12-port vacuum manifold (Sigma-Aldrich Company Ltd., Poole, Dorset, UK). Strata C18 SPE cartridges (3 mL) were purchased from Phenomenex (Macclesfield, Cheshire, UK). De-ionised water was obtained from a Synergy 185 Water Purification System (Merck Millipore Ltd., Watford, Hertfordshire UK).

2.2.3. Preparation of the derivatised mixed prohormone standard

A mixed standard was prepared by adding 20 μ L of each of the 12 prohormones, at an initial concentration of 1 mg/mL in methanol, and then diluting with an equal volume of methanol to give a concentration of each prohormone of 0.04 mg/mL. 100 μ L of this solvent was evaporated to dryness under a gentle stream of nitrogen.

The *tert*-butyldimethylsilyl (TBDMS) derivatising reagent was prepared by the standard HFL method: 18 g of ammonium iodide, 1.5 mL DMF, 3 mL MTBSTFA and 30 mL of ethanethiol were all mixed thoroughly, capped and left for 10 mins.¹ 100 μ L of this solution was then added to the dried prohormones and incubated at 100 °C for 1 hour. Methanol (0.5 mL) and reagent grade water (0.5 mL) were added and whirl mixed. Hexane (2 mL) was then added to the mix. The sample was inverted gently and the top hexane layer was transferred to a small glass tube. The hexane extraction was repeated another two times. The sample was then evaporated under nitrogen gas and reconstituted in 30 μ L of

undecane.¹ As described below, the sample was then analysed by GC/MS in both 1D and 2D-GC modes.

2.2.4. Instrumental parameters used for analysing the derivatised prohormones by gas chromatography/ mass spectrometry (GC/MS)

The GC/MS analyses were performed using a Leco Pegasus® IV time-of-flight (TOF) mass spectrometer (Leco Corporation, St Joseph, Michigan, USA) fitted with an Agilent 6890 gas chromatograph (GC) (Agilent Technologies, Santa Clara, California, USA) modified to house a secondary oven and a twin stage cryo-modulator (Leco Quad-Jet). The GC was equipped with an Agilent split/splitless injector and a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The ionisation source was held at 280 °C and the ionisation energy was set at 70 eV. The MS was calibrated against m/z ions 69, 131, 219 and 502 using perfluorotributylamine (PFTBA). Data processing and mass spectral deconvolution was performed with the Leco ChromaToF® software. The GC analyses were performed in two different modes: one dimension (1D) and two dimensions (2D); as described in the following paragraphs.

2.2.4.1. 1D-GC mode

The gas chromatography separation was performed using a Restek RXi5MS (30 m x 0.25 mm i.d. x 250 µm) (Thames Restex, Sauderton, Buckinghamshire, UK) capillary column in the first dimension and a SGE BPX50 (1.79 m x 0.11 mm x 0.1 µm) (SGE, Milton Keynes, Buckinghamshire, UK) capillary column in the second dimension. This is the traditional non-polar first dimensional column and shorter polar second dimensional column used for comprehensive gas chromatography and is a combination that has successfully been used in

the laboratory by others. The carrier gas used was helium (1.0 mL/min, constant flow). The injector temperature was held at 280 °C, with a 1 µL splitless injection (valve time of 90 s). The main oven temperature programme was 100 °C (1.5 min), 10 °C /min to 340 °C (10.0 min). The secondary oven was operated at 15° C above the primary oven. The modulation time was set for 0 s (effectively the cryogenic modulator was disabled) and all the compounds will therefore elute directly into the mass spectrometer. The TOF mass spectrometer was operated at 6 spectra/s acquisition rate in the mass range: 45 to 550 Da.

2.2.4.2. 2D-GC mode

The operating conditions in the 2D-GC mode were identical to those used in the 1D-GC mode, except for the modulation time set at 4 s, and the TOF mass spectrometer being operated at an acquisition rate of 200 spectra/s, in the mass range: 45 to 550 Da. As is the convention, the acquisition rate was optimised to provide at least 20 data points across a peak. Since the width of the analyte peaks obtained in 2D mode are significantly narrower than in 1D mode, following cryogenic modulation, the acquisition rate needs to be significantly higher than that used in the 1D mode.

2.2.5. Preparation of the underivatised prohormone standard mix

100 µL of each prohormone solution (10 µg/mL, in methanol), provided by HFL Sport Science, was added to a 2 mL autosampler vial which gives a final concentration of each prohormone of 833 ng/mL and a total volume of 1200 µL. 500 µL of the mixed standard was dried and reconstituted in the same volume of acetonitrile.

2.2.6. Instrumental parameters used for analysing underivatised steroids by comprehensive gas chromatography / mass spectrometry (GCxGC/MS)

Underivatised prohormones were resolved using comprehensive gas chromatography. As previously reported, this was achieved through the use of an Agilent 6890 GC, modified to house a secondary oven and twin-stage cryo-modulator. Cryo-cooling was enabled by nitrogen gas passing through a liquid nitrogen dewar. The GC was equipped with an Agilent split/splitless injection port and a CombiPAL autosampler. The separation was performed using a J&W DB-5ms (30 m \times 0.25 mm \times 0.25 μ m; Agilent Technologies) capillary column in the first phase and an SGE BPX50 (2 m \times 0.1 mm \times 0.1 μ m; SGE, Milton Keynes, UK) in the second phase. The carrier gas used was helium (Air Products, BIP Grade) in constant flow mode (1.0 mL/min). The modulation time was set at 6 s with the period of the hot pulses set at 0.6 s.

The samples were injected onto a splitless liner (3 mm internal diameter). The injector temperature was held isothermally at 220 $^{\circ}$ C for a 1 μ L splitless injection (valve time of 90 s). The main oven temperature programme was 65 $^{\circ}$ C (90 s), 15 $^{\circ}$ C/min to 290 $^{\circ}$ C (15 min). Over the entire run, the secondary oven was maintained 15 $^{\circ}$ C higher than the main oven and the modulator temperature was kept 30 $^{\circ}$ C higher than the main oven.

The analysis was performed using a Leco® Pegasus® IV time- of-flight (TOF) mass spectrometer. The TOF mass spectrometer provided unit-resolution in the mass range 5-1000 Da. The ion source temperature was 230 $^{\circ}$ C and the ionisation energy was 70 eV (EI). The TOF mass spectrometer was operated with an acquisition rate of 200 Hz. A solvent delay of 500 s was used. The data processing and mass spectral deconvolution were performed with the Leco® ChromaToF® software.

2.2.7. Validation studies for the analysis of underivatised steroids by comprehensive gas chromatography / mass spectrometry (GCxGC/MS)

The linearity of the GC/MS response for each of the steroids in acetonitrile was determined. A mixed standard, in acetonitrile, was used to prepare a 5-point calibration curve at concentrations of 500, 200, 150, 125 and 50 ng/mL for each steroid. Based on a 1 μ L splitless injection of each solution, this is equivalent to a range of 500 - 50 pg being injected on column.

The limits of detection (LOD) and limits of quantitation (LOQ) were calculated by repeat injections (x10) of 1 μ L of the lowest concentration standard (50 ng/mL), equal to 50 pg on column. The limit of detection was taken as a value that was 3 x the standard deviation determined at this lowest concentration; whereas the limit of quantitation was taken as a value of 10 x the standard deviation.

2.2.8. Preparation and characterisation of the molecular imprinted polymers

Molecular imprinted polymers can be developed to achieve selective binding for a family of similar compounds. A variety of potential polymer “recipes” were therefore investigated, using the literature as a guide. Gadzala-Kopciuch used progesterone, testosterone and 17 β -estradiol as templates to develop SPE cartridges for clean-up and detection of steroids in urine using LC. They reported good results and clear sample clean-up.² Doue *et al.*, used 17 β -estradiol as a template for analysis of 12 steroids in cattle urine.³ SPE was used as a clean-up before GC-MS analysis. Satisfactory recoveries (65%+) were recorded for all compounds. This is similar to the work described here in that one template is used for several compounds. We sought to improve on the detection protocol by the use of GCxGC-MS. A number of different monomer systems (2-vinylpyridine, 4-vinylpyridine,

hydroxyethylmethacrylate, methacrylic acid) were considered, as were the crosslinkers: ethylene glycol dimethacrylate and divinylbenzene. Finally, based on the available literature and strongly guided by the experience of Dr Nicholas Turner in this field, MAA and 4-VP (monomers) and TRIM (crosslinker) were selected for study. The relative uptake of each of these was studied for both the MIP (testosterone imprinted polymer) and NIP (a reference non-imprinted polymer).

Testosterone was used as a template for three reasons. Firstly, it is one of the simplest steroidal structures and its chemistry is well understood. Secondly, many of the “designer” steroids that are used in doping aim to mimic the functionality of testosterone, so this molecule should potentially act as a good surrogate template. Finally, as testosterone is an endogenous steroid it is not analysed in the same way as the prohormone compounds of interest (such as nandrolone). By using testosterone as the surrogate template we can reduce the risk of false positives that may be caused by leaching of the template.

The prohormone compounds selected for study share a high level of similarity to testosterone, in terms of structure, charge and the functional groups present. These similarities increase the probability that a MIP developed for a close analogue will bind to all targets, but also offer the opportunity to demonstrate the viability of the developed technique to separate and quantify structurally similar compounds.

For the polymerizing procedure, the template (testosterone), the monomers (MAA or 4-VP), cross-linker (TRIM) and free-radical initiator (AIBN) were dissolved in the porogen solvent, chloroform, in the molar ratio 1:4:20. A quantity of 20.0 mg of testosterone was dissolved into 1.510 mL of chloroform in a glass vial (4.5 mL). To this solution, 23.5 μ L of methacrylic acid (monomer) and 1.745 mL of TRIM were added and mixed by

vortexing. AIBN (10 mg) was then added to this solution and again mixed via vortexing. Once all the components were dissolved, the solution was sparged with oxygen-free nitrogen for 1 minute and the vial sealed. Polymerization was achieved by heating the solution in the sealed glass vial, in a dry oven, at 60 °C for 24 hours.⁴

After polymerization, the vials were smashed and the monolithic polymers obtained were ground in a mortar and pestle and wet-sieved through a mesh metal sieve. Fine particles were removed by repeated sedimentation using acetonitrile and up to 2 litres of acetonitrile was required for this procedure. A fraction with particle sizes of 38–63 µm was then collected. At least four repeated grinding and sieving cycles were necessary over 7 to 10 days, to reduce the size of the particles and to obtain an adequate amount of particles for packing into the cartridges.

Removal of the imprinted testosterone from the MIPs particles was undertaken by a Soxhlet extraction with methanol–acetic acid (9:1, v/v) for 48 hours, this period had previously optimised within the group. Non-imprinted polymers (NIPs) were synthesized and prepared simultaneously, under the same conditions but without adding the testosterone template.

Six empty SPE cartridges (3 mL) were packed with 20 mg of MIPs or NIPs (3 of each). This was undertaken by placing a frit carefully into the empty cartridge, weighing the amount of MIP, and then adding an additional frit. The MIPs extraction was performed on a 12-port SPE vacuum manifold. The polymers were conditioned prior to use with methanol (1 mL) and with water (1 mL).

The two molecular imprinted polymers generated were named M5 (MAA) and M6 (4-VP), respectively. Similarly, the non-imprinted polymers were named B5 (MAA) and B6 (4-VP), respectively.

Scanning electron microscope images were recorded using secondary and backscatter electron detectors on an FEI Quanta 200 3D, with a tungsten filament source operating at an accelerating voltage of 15 kV, beam current 0.5 nA and a 15 mm working distance.

2.2.9. An evaluation method to determine the percentage recovery of underivatized prohormones from synthetic urine using MIPs

The recovery of the analytes was determined by comparison of peak areas from synthetic urine samples spiked with known amounts of steroids (10 ng/mL), processed according to the described method versus non-extracted pure standards which represent 100% recovery. As for the sections above, the response factors (determined as peak areas) of each of the steroids were determined from 1 μ L splitless injections of a mixed standard in acetonitrile. The polymers were conditioned with methanol (1 mL) and then with water (1 mL). 1 mL of synthetic urine was spiked at the 10 ng/mL level for each prohormone. 1 mL of buffer, at pH 9, was then added and the 2 mL solution was whirl-mixed three times before being added to each of the MIPs cartridges. The spiked, buffered urine samples were loaded onto the MIPs cartridges in three steps (100 μ L, 900 μ L and 1000 μ L). The washing step was then performed with 1 mL of water and 1 mL of 40 % methanol in water (v/v).

Elution of the extracted analyte was achieved by adding 1 mL of pure methanol. The load, wash and elution solvents were collected in 70 mm glass tubes. The elution solvents were then transferred to a 2 mL autosampler vial and each was evaporated using a SF50 (Genevac). Once dry they were then reconstituted in 20 μ L of acetonitrile and the solution of underivatized hormones were injected in the comprehensive gas chromatography system. Urine samples were prepared in triplicate at each concentration. Specificity was demonstrated by analysis of blank urine samples.

2.2.10. Further validation of the GCxGC-MS method when applied to prohormones and designer steroids

The screening method developed and optimised for the detection of the 12 prohormones, was evaluated for its ability to detect and identify a wider range of steroids present in urine samples, including several designer steroids. A request for epitestosterone, methyl testosterone and a range of “designer steroids” was made to HFL Sport Science. As a result, seven additional steroids were provided for the study. These included: methyl testosterone, epitestosterone, Xtren, trenazone, gestrinone, tetrahydrogestrinone, and epistane. Methyl testosterone was used as an internal standard. The solvents, reagents and materials prepared for the initial prohormone study were once again utilised in this study.

Stock solutions of the six designer steroids were prepared in methanol, at a concentration of 100 µg/mL. 100 µL aliquots of the six designer steroids were then combined and added to 400 µL of MeOH, to make a mixed designer steroid stock solution, each at a concentration of 10 µg/mL. This was further diluted with methanol to generate a second mixed designer steroid stock solution at 1 µg/mL. The stock solution was then diluted in methanol to produce DS#1, with each steroid present at a concentration of 0.5 µg/mL.

Combining together 500 µL of DS#1 with an equal volume of a mixed prohormone stock standard (at 500 ng/mL) resulted in a mixed designer and prohormone standard (DSP#1) with each steroid present at a concentration of 250 ng/mL.

An internal standard solution was prepared in methanol, with methyltestosterone at a concentration of 500 ng/mL. The dilution of the above mixed standards with varying amounts of methanol, followed by the dilution of these intermediary solutions with a

consistent 1:3 mix of the internal standard solution resulted in a range of mixed designer/prohormone solutions, with the internal standard present at a concentration of 167 ng/mL in each. DSPI#1 contains the prohormones and designer steroids, each at a concentration of 100 ng/mL; whereas DSPI#2 and DSPI#3 contain each of the designer steroids and prohormones at concentrations of 50 and 25 ng/mL, respectively.

2.2.11. A method to evaluate the performance of the MIPs screening technique, when applied to urine samples containing designer steroids (10 ng/mL) in the presence of prohormones

1 mL samples of synthetic urine were spiked with designer steroids and an internal standard (methyl testosterone) at 10 and 500 ng/mL of each, respectively. The urine samples were also spiked with the 12 prohormones, over a range of concentrations (2 to 10 ng/mL), to ascertain whether their presence influenced the measurement of the designer steroids. The solution therefore contained a total of 19 steroids, to which 1 mL of buffer, at pH 9, was added and whirl-mixed three times.

As before, the polymers were conditioned with methanol (1 mL) and then with water (1 mL). The spiked urine samples were loaded on the MIPs cartridges in three steps (100 μ L, 900 μ L and 1000 μ L). The washing step was then performed with 1 mL of water and 1 mL of 40 % methanol in water (v/v).

Elution of the extracted analyte was achieved by adding 1 mL of pure methanol. The load, wash and elution solvents were collected in 70 mm glass tubes. The elution solvents were then transferred to a 2 mL autosampler vial and each was evaporated using a SF50 (Genevac). Once dry they were then reconstituted in 20 μ L of acetonitrile.

It should be noted that since conducting these experiments the WADA specifications for anabolic androgenic steroids have changed. In 2013 it reduced to 5 ng/mL, from a level of 10 ng/mL in 2010.^{5,6}

The level of recovery for the designer steroids was determined by the use of standard solutions that mimicked the process that the urine samples experienced, without the addition to urine and MIPs extraction. These standards bracketed the MIPs samples analysed on the GCxGC/MS. The average peak area obtained for each designer steroid in these standards was divided by the peak area of the internal standard. The percentage recovery in each of the MIPs cartridges was then calculated from the ratio of peak areas for each designer steroid relative to the peak area for the internal standard divided by the values in the bracketed controls.

2.3. A pilot study to evaluate the feasibility of the analysis of estradiol by GC/MS and LC/MS/MS following extraction by aptamers

2.3.1. Materials

The DNA aptamer (5-Biotin GCTTCCAGCTTATTGAATTACACGCAGAGGGTAGCG-GCTCTGCGCATTCAATTGCTGCGCGCTGAAGCGCGGAAGC-3) was purchased from Integrated DNA Technology Inc. (Leuven, Belgium). De-ionised water was obtained from a Synergy 185 Water Purification System (Merck Millipore Ltd., Watford, Hertfordshire, UK). Estradiol was purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Tris-HCl, NaCl, EDTA, MgCl₂, and urea were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). N-*tert*-butyldimethylsilyl - N-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethyl chlorosilane (TBMCS) was purchased from

Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Solvents (HPLC Grade) were purchased from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK).

2.3.2. Apparatus and equipment

Vortexing was performed using the MS3 Basic (IKA, Staufen, Germany). The magnetic beads (Dynabeads M-280) and the magnetic block were purchased from Invitrogen (Oslo, Norway). Centrifugal evaporation was performed using a SF50 and CVP100 pump (Genevac Ltd., Ipswich, Suffolk, UK). The autoclaving of water was performed using an Autoclave 2100 Series (Prestige Medical Ltd., Blackburn, UK). Heating was performed in Techne DB-3A dry block heater (Bibby Scientific, Staffordshire, UK). Eppendorf tubes were obtained from the central stores. Glass vials were obtained from Chromacol Ltd. (Welwyn Garden City, Herts, UK).

2.3.3. Preparation of the GC/MS calibration standards

Estradiol standards were prepared by weighing the solid material and dissolving in methanol. Concentrations of 14,286 µg/mL, 1428.6 µg/mL, 142.86 µg/mL, 1.4286 µg/mL, 0.14286 µg/mL were used to prepare a calibration curve. A volume of 100 µL of the sample was transferred to a 2 mL glass vial and dried using a Genevac SF50 centrifugal evaporator with CVP100 pump. 100 µL of the derivatising reagent (MTBSTFA with 1% TBDMCS) was then added and incubated at 100° C, for 1 hour, in a Techne DB-3A dry block heater. Of this, 1 µL aliquots were then injected into the GC system

2.3.4. Optimised parameters used for the GC/MS

The analytical instrument used in this study is a Leco Pegasus® IV time-of-flight (TOF) mass spectrometer (Leco Corporation, St Joseph, Michigan, USA) fitted with an Agilent 6890 gas chromatograph (GC), (Agilent Technologies, Santa Clara, California, USA) modified to house a secondary oven and a twin stage cryo-modulator (Leco Quad-Jet). The analyses were performed in 1 D-GC mode and a secondary oven was fitted but the cryo-modulator was not operated. The GC was equipped with an Agilent split/splitless injector and a CTC CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The chromatographic separation was performed using a SGE BPX50 (30 m x 0.25 mm i.d. x 250 µm film thickness) (SGE, Milton Keynes, Buckinghamshire, UK) capillary column and a SGE BPX5 (2m x 0.1mm x 0.1µm; SGE UK) second capillary column. The carrier gas used was helium (Air Products, BIP Grade, 1.0 mL/min, constant flow). The injector temperature was held at 280 °C, with a 1 µL splitless injection (valve time of 90 s). The main oven temperature programme was 200 °C (1.50 min), 6 °C /min to 320 °C (4.80 min). The TOF mass spectrometer was operated at 10 spectra/s acquisition rate in the mass range: 50 to 650 Da. The ionisation source was held at 280°C and the ionisation energy was set at 70 eV. The MS was calibrated against *m/z* ions 69, 131, 219 and 502 using perfluorotributylamine (PFTBA). Data processing and mass spectral deconvolution were performed with the Leco ChromaToF® software.

2.3.5. Preparation of the LC/MS/MS calibration standards

The initial mobile phase (5% MeOH / 95% deionised water) was spiked with estradiol over a range of concentrations (100 ng/mL, 10 ng/mL, 1ng/mL, 100 pg/mL). A calibration curve for estradiol was obtained by assigning the concentration of estradiol to *x* and the peak area

of estradiol to y , respectively. Subsequently, linear regression was performed over the estradiol concentration range of 100 ng/mL to 100 pg/mL.

2.3.6. Optimised parameters used for the LC/MS/MS

A TSQ Quantum LC/MS/MS (Thermo Electron Corporation), equipped with an electron spray ionisation (ESI) source and a Surveyor HPLC system was employed for the evaluation of the efficacy of the estradiol extraction from the aptamer. The two columns evaluated were:

1. Hypersil GOLD, C₁₈ Column (150 x 4.6 mm, 5 μ m) connected to a C₁₈ guard-cartridge, in a guard-cartridge holder, from Thermo-Fisher;
2. XBridge, C₁₈ Column (50 x 2.1 mm, 2.5 μ m) from Waters.

Data acquisition was performed in negative ion mode, using the transitions:

m/z 271 \rightarrow 145, m/z 271 \rightarrow 183, m/z 271 \rightarrow 158 and m/z 271 \rightarrow 158.

The ions were selected by the automated Xcaliber software, determined as the ions present with the highest abundance. The mobile phase conditions were optimised, the lowest baseline noise was achieved by using a gradient consisting of water (Line A) and methanol (Line B) separated on a XBridge C₁₈ Column, at a flow rate of 200 μ L/min. The gradient of the mobile phase started with 5 % methanol for 2 mins, it was then ramped up to 90 % methanol over 12 mins and maintained at 90 % methanol for 3 mins, before being returned to the initial conditions. The initial condition used is highly aqueous, greater interaction of the non-polar 17 β -estradiol with the C₁₈ Column is therefore expected. Ramping the mobile phase to a more organic condition, using methanol, will result in the elution of the 17 β -

estradiol. The ESI-MS/MS conditions were: spray voltage (4000 V); sheath gas (nitrogen, 10 arbitrary units of gas pressure); auxiliary gas (nitrogen, 10 arbitrary units of gas pressure); ion transfer capillary temperature (330 °C); collision gas (argon, 1.5 mTorr gas pressure). The negative ion polarity injection volume was configurable from 10-50 μ L.

2.3.7. Method for binding the aptamer onto the magnetic beads

Following the manufacturer's protocol, the magnetic beads (Dynabeads M-280, coated with streptavidin) were first washed with a Binding and Washing buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl) before use.⁷ After the trapping of the washed beads on to the magnet, the supernatant was removed and the beads were re-suspended in the same volume as originally added. A volume of 100 μ L of the beads suspension was then introduced into an Eppendorf tube containing 50 μ L of Binding and Washing buffer and 50 μ L of aptamer solution (2 μ M). After 15 minutes of vortexing, the tube was placed on a magnetic block to allow the collection and separation of the beads; the supernatant was then removed and the beads were washed twice with the Binding and Washing buffer.

The binding process was confirmed by measuring a change in the UV spectrum (scanned between 200 and 300 nm) transmitted through the solution before and after exposure to the beads. Before adding the beads, the aptamer solution showed an absorbance at $\lambda_{\text{max}} = 260$ nm. After the above procedure, there was no absorbance at $\lambda_{\text{max}} = 260$ nm, indicating that the aptamer had bound to the magnetic beads.

2.3.8. An overview of the method for evaluating the performance of the aptamer coated on to magnetic beads

As previously discussed, effective sample clean-up strategies and efficient chromatographic separations are required for the elimination of matrix interference to enable target species to be detected and quantified in complex biological samples. However, clean-up methods that require a large number of steps can lead to analyte loss. The bead-based method shown in Figure 2.1 only requires the following steps:

- i) the estradiol is spiked into the Hormone Binding Buffer;
- ii) 100 μL of this spiked buffer is added to the vial and vortexed with the aptamer coated magnetic beads for 1 hour;
- iii) the beads are magnetized and the supernatant is decanted and prepared for GC/MS and/or LC/MS/MS analysis;
- iv) the magnet is removed and an Elution Buffer (100 μL) is added and vortexed for 20 minutes;
- v) the beads are magnetized and the Elution Buffer supernatant is removed and prepared for GC/MS and/or LC/MS/MS analysis.

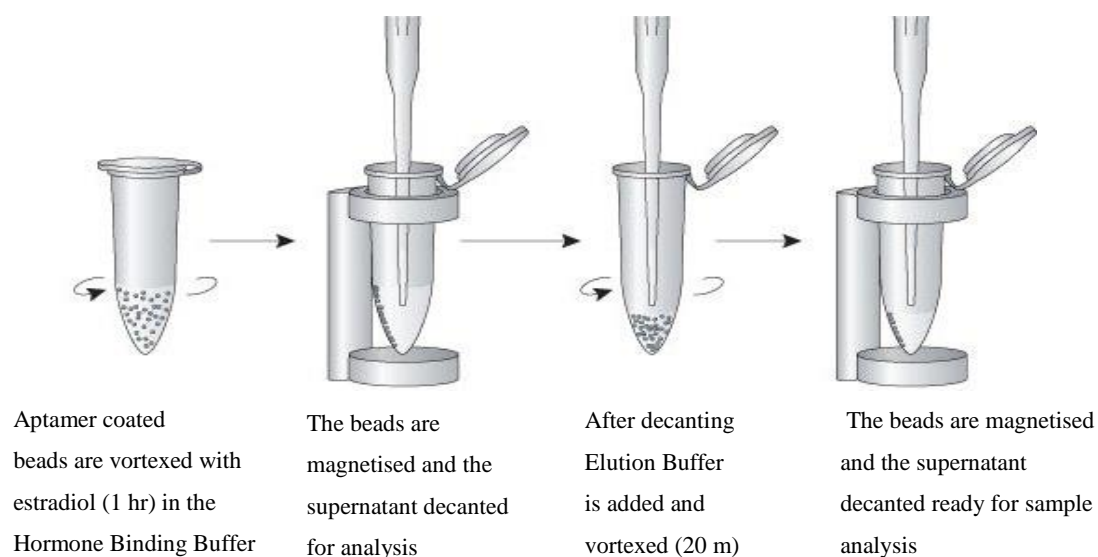


Figure 2.1 A schematic representation of the experimental procedure, showing the generation of the various solutions that were then prepared for analysis to evaluate the performance of the procedure.

The various buffers used during the study were prepared following a review of the literature and their compositions are listed below: ⁸

- *Hormone Binding* : 0.454 mM estradiol, 100 mM Tris-HCl, 200 mM NaCl, 25 mM KCl, 10 mM MgCl₂, 5% ethanol, pH 8.0
- *Elution (#1)*: 40 mM Tris-HCl (pH 8), 10 mM EDTA, 3.5 M urea;
- *Elution (#2)*: 10 % sodium dodecyl sulphate (SDS).

2.3.9. A GC/MS method for the evaluation of the binding efficiency of the aptamer for estradiol and the efficiency of the Elution Buffer

A 100 μ L suspension containing the aptamer coated beads, was magnetised and the supernatant removed. 100 μ L of the Hormone Binding solution, containing estradiol at

0.459 mM, was then added and vortexed for 1 hour, at room temperature. The beads were then magnetically separated and the supernatant was removed by pipette and diluted up to 1 mL in distilled water, to be prepared for GC/MS analysis. Liquid-liquid extraction, using 1 mL of hexane, was undertaken by inverting the 10 mL screw top glass vial ten times. The extracted hexane layer was transferred into a 2 mL autosampler vial with a glass pipette and then dried using a Genevac SF50 centrifugal evaporator with CVP100 pump. 100 μ L of the derivatising reagent (MTBSTFA with 1% TBDMCS) was then added and incubated at 100° C, for 1 hour, in a Techne DB-3A dry block heater. 1 μ L aliquots were injected into the GC system for analysis.

100 μ L of the Elution Buffer was added to the magnetic beads and vortexed. After 20 minutes, the tube was placed on a magnetic block to allow collection of the beads; the supernatant was then removed and prepared for GC/MS analysis, as above.

2.3.10. A GC/MS method for determination of the percentage recovery of estradiol from the aptamer

For the recovery standard, 100 μ L of the Hormone Binding Buffer (0.454 mM Estradiol, 100 mM Tris-HCl, 200 mM NaCl, 25 mM KCl, 10 mM MgCl₂) was diluted to 1 mL with de-ionised water in a 10 mL screw top glass vial and then this solution was extracted with hexane (1 mL). The liquid-liquid extraction was undertaken by inverting the glass vial ten times. The extracted hexane was then transferred into a 2 mL autosampler vial with a glass pipette and then dried using a Genevac SF50 centrifugal evaporator with CVP100 pump. 100 μ L of the derivatising reagent (MTBSTFA with 1% TBDMCS) was then added and incubated at 100° C, for 1 hour, in a Techne DB-3A dry block heater (Techne). 1 μ L aliquots were injected into the GC system.

2.3.11. Extraction of the buffer for LC/MS/MS analysis

A 100 µL suspension of the Binding Buffer (spiked with 1 ng/mL of estradiol) was diluted to 1 mL with de-ionised water and then extracted with 1 mL of hexane. The extracted hexane was then dried and reconstituted in 100 µL of the mobile phase (90% methanol/10% water). The analysis was repeated three times.

A 100 µL suspension of Binding Buffer spiked with 1 ng/mL of estradiol was diluted to 1 mL with de-ionised water and then extracted with 1 mL of TBE. The extracted TBE was then dried and reconstituted in 100 µL of the mobile phase (90% methanol/10% water). The analysis was repeated three times.

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- 3. The development of a method for the analysis of prohormones by comprehensive gas chromatography/mass spectrometry following extraction by molecular imprinted polymers**

3.1. Introduction

The Olympic drug testing body and WADA have huge a responsibility to ensure the accuracy of its testing processes so that those athletes that are doping are not missed (false-negative) and innocent athletes are not accused incorrectly (false-positive). The use of two test samples helps reduce the number of false-positives; whereas, the use of no-notice testing of athletes, while out of competition, narrows the window of opportunity to achieve benefit from banned substances.¹ Anabolic agents are the most common performance enhancing substances detected in athletes.

As shown in Chapter 1, the steroid structure can be best described as a fused tetracyclic nucleus, modified by the addition or removal of polar or non-polar functional groups and by different levels of saturation of the nucleus. The detection of the anabolic agent can be confounded by the large number of endogenous steroids and metabolites naturally present in low amounts within the complex matrix of the urine.

Another challenge is the sheer number and variety of steroids that have been produced in the laboratories of pharmaceutical companies but discarded during the development stage.² With unpublished side effects, these steroids often find a route in to doping violation and can cause serious harm to the athlete. The development of new designer steroids, that are modified to specifically avoid detection, is also a serious concern.

Furthermore, the requirements for low limits of detection has made unavoidable the use of the selective ion monitoring (SIM) mode on the quadrupole mass spectrometer; which, although highly sensitive, does have limitations when used in a comprehensive screening programme and may be exploited to produce false-negative results. These limitations stem from the fact that the SIM mode utilises a selection of pre-determined ions, from the

expected fragmentation profile of a given steroid or a group of steroids, at given retention times within the chromatogram. If a rogue chemist is aware of these detection windows, following derivatisation, then they can undertake a number of strategies to manipulate the steroid structure. For example, the steroid structure can be modified to introduce steric hindrances, thus preventing the derivatisation process.

Currently, sample preparation includes a number of analytical steps with the overall extraction efficiency and selectivity of the method dependent on the technique used. Early methods of steroid analysis usually involved solvent extraction as the first step.³ The main drawback of liquid–liquid extraction is emulsion formation and the extraction can require the use of a large volume of toxic and flammable solvents. Non-polar solvents are often used to minimize the extraction of interfering compounds in the analyses of non-polar steroids.³ Modern methods for the extraction of steroids from urine usually involve liquid-liquid or solid phase extraction.^{4,5,6,7}

Steroids are present in urine in the conjugated form, because the hydrophilic nature of the conjugated steroid aids urinary excretion. During Phase II metabolism, lipophilic steroids are conjugated with either a glucuronide or sulphate moiety. Steroids in the unconjugated form contribute to less than 3% of the total steroids excreted in urine.^{8,9,10} Steroid androgens are generally conjugated with a glucuronide moiety. Conjugated steroids can disintegrate under the high temperatures used in GC analysis, the first step thus requires de-conjugation of the steroid into its free form. The choice of hydrolysis method is dependent on the steroid and conjugate moiety and hydrolysis conditions are often inefficient. Hydrolysis is often carried out by enzymatic or chemical means. Hydrolysis of a sulphate moiety is analytically more challenging, as many sulfate conjugates are resistant to enzyme hydrolysis or generate unwanted by-products and chemical hydrolysis is not without issue.⁸

Each preparation step in steroid analysis has its own challenges, from the efficient cleavage of the glucuronide or sulphate to the optimized conditions required for derivatisation.⁸ These issues have resulted in the recent focus on direct conjugation analysis of steroids with a glucuronide moiety by LC/MS and LC/MS/(MS), thus removing the need for hydrolysis or derivatisation.

LC/MS/(MS) is often deemed as the solution for all issues related to steroid detection. However, since the optimised mobile phase conditions, the column selected and the prerequisite for steroids to be in ionic form are all dependent on the steroid structure, manipulation of the steroid structure can still be used by the rogue chemist to avoid detection. The relatively poor chromatographic performance of LC, compared to GC, is another issue facing the movement towards LC/MS/(MS) as a solution for the screening for unknown steroids.

The application of LC-MS/(MS) for the detection of steroids remains limited, owing to the the difficulty in ionizing anabolic steroids under ESI conditions, due to the lack of acidic or basic moiety in their chemical structure. The characteristic structure of anabolic steroids can also have a limiting effect in their ionization efficiency. In a recent study, it was shown that steroids which contain a conjugated keto-functional group at C3 showed good proton affinity and stability; generating the $[M+H]^+$ ion as the most abundant precursor ion. Conversely, steroids containing conjugated/unconjugated hydroxyl functional groups at C3, generated multiple ions, and showed poor stability with relatively high LODs.¹¹

It is proposed that a combination of comprehensive gas chromatography/mass spectrometry and existing LC/MS/(MS) methods could narrow the window of opportunity so much so that it becomes a deterrent to rogue chemists, as the challenges involved in designing

steroids that can avoid detection by both techniques will be too great. Comprehensive gas chromatography can provide additional surveillance modes for the screening of new compounds for less effort than LC/MS/MS, since electrospray ionisation and mobile phase condition optimisation are not critical.

Whilst it is widely accepted that GC/MS analyses are traditionally constrained to derivatisable steroids, this chapter will describe the development and evaluation of a new method that will couple bespoke molecular imprinted polymers (MIPs) with comprehensive gas chromatography/time of flight mass spectrometry (GCxGC/MS) for the extraction, separation, identification and quantification of underivatised steroids (equivalent to free or hydrolysed steroids) spiked into synthetic urine. Due to practical and ethical considerations the study of conjugated steroids in urine is beyond the scope of this work. It is recognised that if this technique is to be applied for Sports Testing then it will either need to have the sensitivity to detect unconjugated steroids directly in urine or a hydrolysis technique will need to be developed prior to the application of the MIPs and GCxGC/MS. The study is based on the assumption that the latter will be applicable and this work is thus providing the proof of principle evaluation of the latter stages of the process.

A cohort of twelve steroids were provided by HFL Sport Science for the evaluation of the method. The cohort were selected to capture the diverse range present in endogenous and exogenous anabolic androgenic steroids, their metabolites and prohormones. The structures of the steroids can be found in Figure 1.14 of Chapter 1. As the majority of these steroids are classed as prohormones, the cohort will be referred to collectively as the “prohormones”. For example, 5(10)-estrene-3 β ,17 β -diol, 4-estrene-3 β ,17 β -diol and 5(10)-estrene-3,17-dione are all precursors for nandrolone. Nandrolone is one of the most frequently detected anabolic androgenic agents detected. Its occurrence has consistently

remained high, with 155 adverse analytical findings reported by WADA accredited laboratories in 2009, contributing to 4.7 % of the total anabolic steroids detected.¹² In 2010, this increased to 250 occurrence, an overall contribution to 7.4 %, making it the second highest detected AAS after stanozolol.¹³ In 2011, data published by WADA showed a similar result with 240 occurrences, an overall contribution of 7.2 %.¹⁴ The popularity of nandrolone as an anabolic agent amongst athletes is mostly related to the structural modification providing favourable anabolic properties while at the same time reducing the androgenic properties.

Initially, this chapter will report and discuss the issues encountered when analysing a mixture of the twelve prohormones, following derivatisation, using both a single GC column and comprehensive chromatography.

It will then characterise and evaluate the performance (chromatographic resolution, linearity, limit of detection and limit of quantitation) of the comprehensive chromatography (GCxGC/MS) method with underivatised steroid standards, containing the twelve prohormones. The quantity of each of the steroids analysed will be representative of the detection levels required by the WADA in urine samples. In 2010 WADA's Prohibitive List specified that hormones should be detectable at the 10 ng/mL level. During the project, the MRPL was subsequently reduced to 5 ng/mL. One of the hormones methyltestosterone, used as an internal standard in Chapter 4, has a lower detection level of 2 ng/mL.

The performance of two non-covalently bonded molecular imprinted polymers (MIPs) and two non-imprinted molecular polymers (NIPs), that were developed to selectively extract a range of steroids from urine, will then be evaluated. Testosterone has been used as the template molecule, with the view that once it is removed the cavities generated will be

complementary in shape, size, affinity and functionality and will therefore bind to a wide range of endogenous androgens, 19-nor-steroids, metabolites, prohormones and synthetic analogues of testosterone (designer steroids). Scanning electron microscope images of the wet-sieved molecular imprinted polymers, templated with testosterone during this project, will be provided and discussed.

Although the method will be used for the extraction of steroids from spiked synthetic urine it is anticipated that in the future it may also be applied to different biological and food samples such as human serum, contaminated meat and nutritional supplements with slight modification to accommodate solid sample analysis.

3.2. Results and Discussion

3.2.1. Chromatographic resolution of derivatised prohormone standards

A standard solution, containing a mixture of the twelve prohormones (40 µg/mL of each in methanol), was prepared and the analytes were derivatised, as described in Section 2.2.3 to provide a solution with 260 ng/µL of each derivatised hormone in undecane (assuming completed conversion). The derivatised prohormones were analysed on the Leco Pegasus IV GCxGC-TOFMS in both 1D- and 2D-GC modes, using the gas chromatography and mass spectrometry parameters summarised in Section 2.2.4. Table 3.1 summarises the prohormones present in the initial mixture and the derivatised species identified, along with their diagnostic ions and their retention times on a single GC column.

Table 3.1. Summary of the key parameters determined for each of the derivatised prohormones analysed in the 1D- GC mode.

Steroid Number	Prohormone	Derivatised Species Identified	Quantitation Ion, m/z (Th)	First Dimension Retention Time (s)
1	5(10)-estrene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	447	1145.77
2	4-estrene-3b,17b-diol	Not detectable	-	-
3	5(10)-estrene-3,17-dione	bis-(O-tert.-butyldimethylsilyl)-	500	1212.58
4	4-androstene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	462	1192.09
5	DHEA	Not detectable	-	-
6	5(6)-androstene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	462	1224.24
7	5a-androstane-3,17-diol	bis-(O-tert.-butyldimethylsilyl)-	461	1208.25
8	5a-androstane-3,17-dione	Underivatised	217	1024.99
9	Nandrolone	bis-(O-tert.-butyldimethylsilyl)-	502	1211.9
10	5(6)-androstene-3,17-dione	Underivatised	286	1045.48
11	Testosterone	bis-(O-tert.-butyldimethylsilyl)-	459	1231.07
12	1,4-androstadiene-3,17-dione	Not detectable	-	-

Potential mechanisms for the generation of the fragment ions in the mass spectra of each of the derivatised prohormones are provided in Appendix A1.1. Derivatisation introduces the tert-butyldimethylsilyl as a functional group replacing either, or both, the hydroxyl or ketone group at both ends of the prohormones structure, depending on the individual prohormone. Its presence inevitably changes the fragmentation pathways, when compared

to those of the native prohormones (Appendix A1.2). The most common fragmentation of the derivatised prohormones is the loss of tert-butyl group (57 Da), as exemplified by 4-androstene-3b,17b-diol,bis-(0-tert.-butyldimethylsilyl). A one step cleavage of the (0-tert.-butyldimethylsilyl) group (132 Da) results in a fragment forming at m/z 387. The fragmentation is most commonly initiated at the D ring, perhaps due to the ring constraint. The subsequent loss of H_2 group results in an ion forming at m/z 384. Fragmentation at the A ring of the derivatised prohormone is accompanied with a rearrangement, resulting in the loss of m/z 56 and reattachment of H group to the silyl group.

Figure 3.1 illustrates the chromatogram obtained for the derivatised prohormones. The numbering of the peaks is consistent with the numbering of the steroids in Table 3.1. It is clear from the abundance responses that it would have been preferable to have diluted the sample prior to analysis, and this may have contributed to some of the loss of chromatographic resolution discussed.

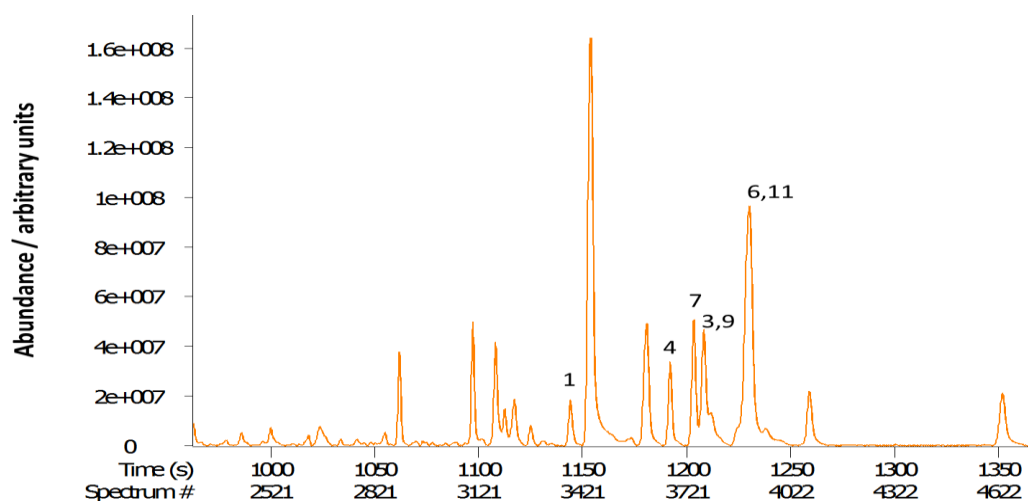


Figure 3.1. Chromatogram illustrating the retention times of the derivatised prohormone species determined in the total ion chromatogram (TIC) in 1D-GC mode. The retention times for the derivatised versions of [1] 5(10)-estrene-3b,17b-diol, [3] 5(10)-estrene-3,17-dione, [4] 4-androstene-3b,17b-diol, [6] 5(6)-androstene-3b,17b-diol, [7] 5a-androstane-3,17-diol, [9] nandrolone and [11] testosterone are labelled on the chromatogram.

Figure 3.2 illustrates the chromatogram obtained and identifies the elution regions for both derivatised and underivatised prohormones.

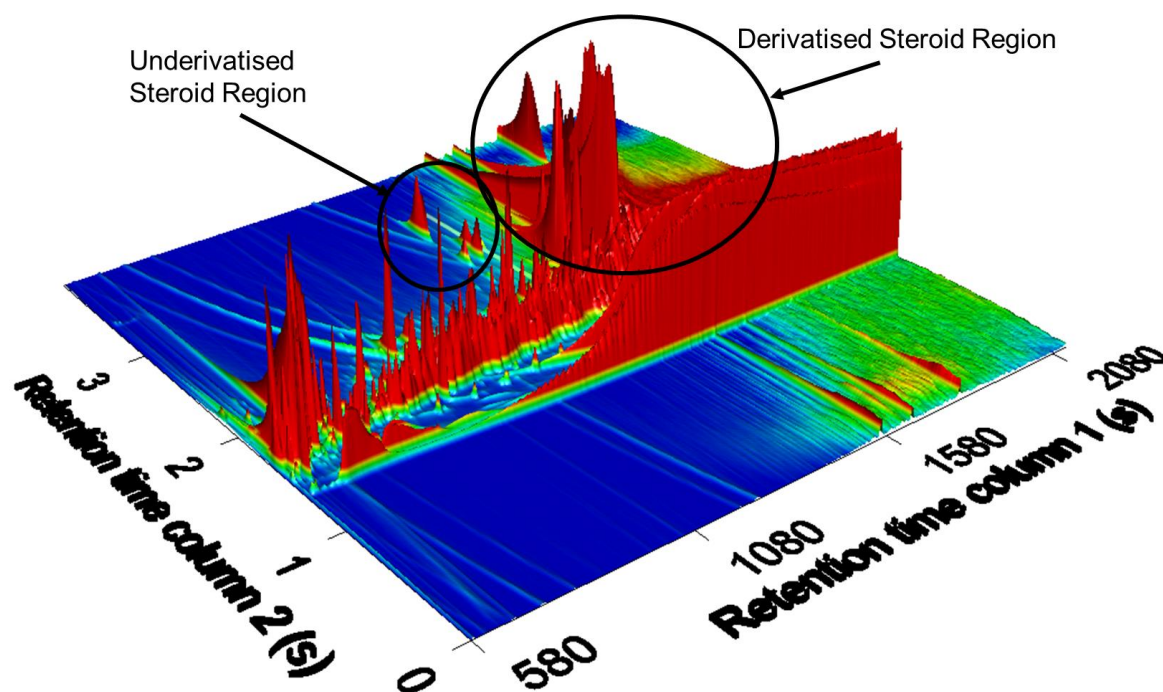


Figure 3.2. A 2D surface plot (TIC) illustrating the retention regions for the derivatised and underivatised prohormone species and their relative location with respect to matrix species.

Table 3.2 summarises the derivatised prohormone species identified, their diagnostic ion and their retention times, following analysis by comprehensive gas chromatography.

Table 3.2. Summary of the first- and second- dimension retention times and diagnostic ion of the derivatised prohormones species identified in the 2D-GC mode.

Steroid Number	Prohormone	Derivatised Species	Quantitation Ion, m/z (Th)	First Dimension Retention Time (s)	Second Dimension Retention Time (s)
1	5(10)-estrene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	447	1588	2.260
2	4-estrene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	447	1548	2.100
3	5(10)-estrene-3,17-dione	bis-(O-tert.-butyldimethylsilyl)- & underivatised	500	1628	2.605
4	4-androstene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	462	1640	2.475
5	DHEA	Not detectable	-	-	-
6	5(6)-androstene-3b,17b-diol	bis-(O-tert.-butyldimethylsilyl)-	462	1644	2.555
7	5a-androstane-3,17-diol	bis-(O-tert.-butyldimethylsilyl)- & underivatised	461	1648	2.595
8	5a-androstane-3,17-dione	Underivatised	217	1368	2.795
9	Nandrolone	bis-(O-tert.-butyldimethylsilyl)-	502	1624	2.475
10	5(6)-androstene-3,17-dione	Underivatised	286	1408	2.865
11	Testosterone	bis-(O-tert.-butyldimethylsilyl)-	459	1648	2.655
12	1,4-androstadiene-3,17-dione	Underivatised	122	1420	2.935

It is clear from the total ion chromatogram shown in Figure 3.2 that the derivatised and underivatised prohormones elute in different regions of the 2D surface plot. The underivatised species, mainly diones, elute together in one location and the derivatised species in another. The main difference is that the derivatised species are retained in the first dimension column for longer, indicating that they have a lower partition coefficient into the mobile phase, which suggests that they either have greater interaction with the stationary phase or lower volatility than the underivatised diones (5(10)-estrene-3,17-dione, 5 α -androstane-3,17-dione, 5(6)-androstene-3,17-dione, 1,4-androstadiene-3,17-dione) and the saturated, 5 α -androstane-3,17-diol.

It is also clear from the total ion chromatogram that it is difficult to resolve the derivatised species from each other and the significant levels of matrix species present. Figure 3.3 illustrates how the derivatised prohormones region can be resolved from other species through the use of their diagnostic fragmentation ions (m/z = 447, 461, 463). Whereas, Figure 3.4 provides an expanded view of the same 2D surface plot, which allows the individual prohormones to be uniquely identified.

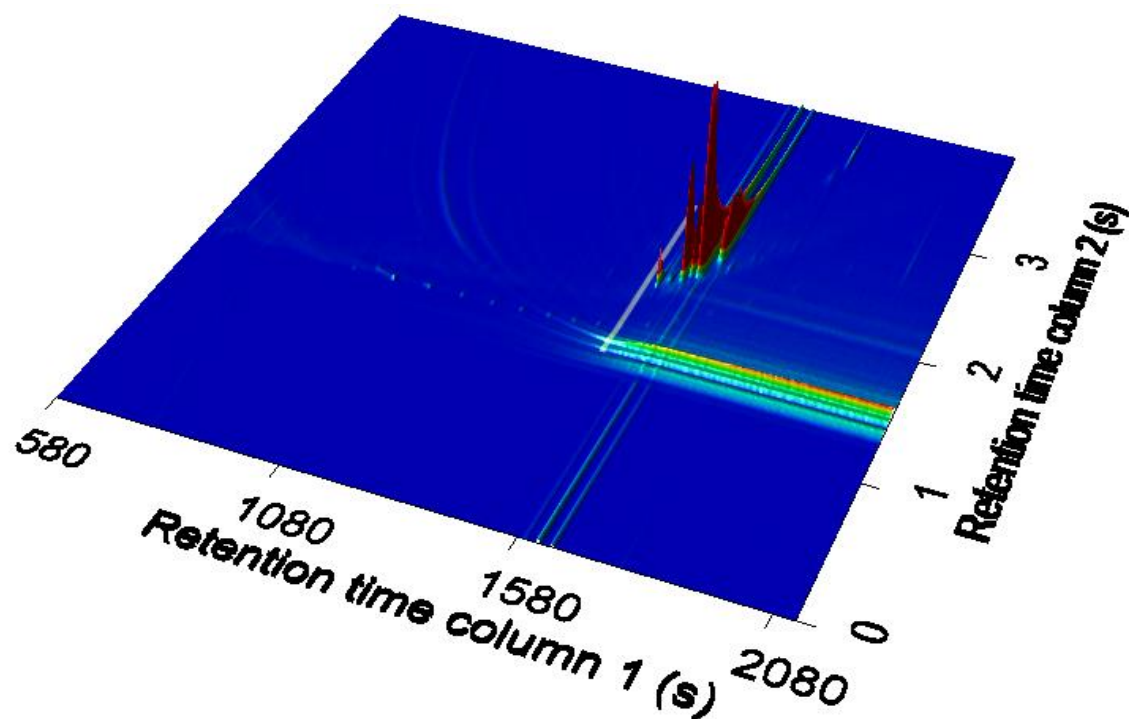


Figure 3.3. A 2D surface plot for the extracted ions ($m/z=447, 461$ and 463) illustrating the retention times of the derivatised prohormone in both dimensions.

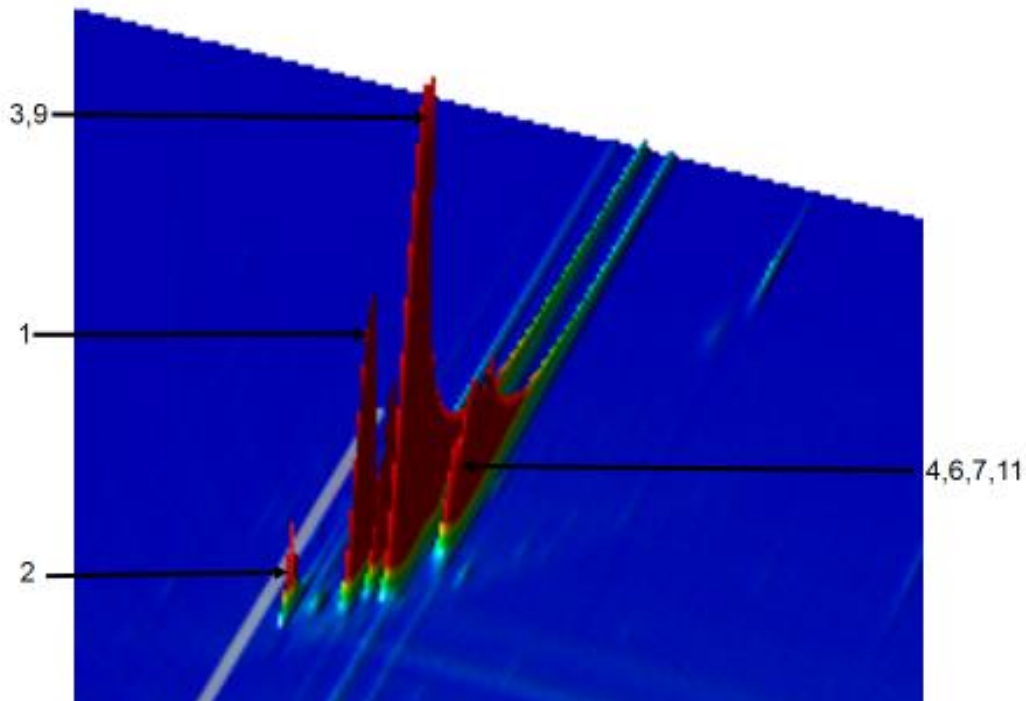


Figure 3.4. Expanded view of the previous 2D surface plot for the extracted ions ($m/z=447, 461$ and 463) illustrating the limited separation of the derivatised prohormones.

The results obtained in both chromatographic modes confirm the previous findings of HFL Sport Science, using standard one-dimensional chromatography; namely, that for the wide range of prohormones that need to be screened, derivatisation does not provide a complete solution. DHEA (5(6)-androstene-3 β -ol-17-one) was not detectable in either chromatographic mode of operation, whereas nandrolone (17 β -hydroxyestra-4-en-3-one) and testosterone (4-androstene-17-ol-3-one) were both detected as the derivatised species. In contrast to the other prohormones, which are all either diols or diones, these three compounds have both a hydroxyl and carbonyl group. Nandrolone and testosterone have the hydroxyl group at the 17-position whereas DHEA has it at the 3-position. DHEA is also unsaturated at the 5-position in Ring B, in contrast to nandrolone and testosterone which are unsaturated at the 4-position in Ring A. 4-estrene-3 β ,17 β -diol (derivatised) and 1,4-androstadiene-3,17-dione (underivatised) were not detected in 1D mode, but were found in 2D mode. As can be seen in Figure 3.2, the application of the second dimension column pulls the prohormones away from most of the matrix species, present as a result of the derivatisation process, placing them in a cleaner chromatographic region. However, as shown in Figures 3.3 and 3.4, it is the coupling of this temporal, chromatographic resolution with the high acquisition rate of the TOFMS that enables the prohormones to be fully resolved using their diagnostic fragmentation ions.

Certain hormones are found derivatised (5(10)-estrene-3 β ,17 β -diol, 4-estrene-3 β ,17 β -diol, 4-androstene-3 β ,17 β -diol, 5(6)-androstene-3 β ,17 β -diol, nandrolone, testosterone), others are found both in the derivatised and underivatised forms (5(10)-estrene-3,17-dione, 5 α -androstane-3,17-diol) whilst some others are not derivatised at all (5 α -androstane-3,17-dione, 5(6)-androstene-3,17-dione, 1,4-androstadiene-3,17-dione). Taking account of the conditions used, some basic relationships can be deduced from these findings:

- i. underivatised species elute significantly earlier than derivatised species;
- ii. diols and species with a hydroxyl group at the 17- position (*e.g.* nandrolone and testosterone) will, in the main, derivatise;
- iii. diones, will, in the main, not derivatise;
- iv. there are exceptions to the above rules and therefore each compound must be evaluated individually.

It was known that derivatisation adds time and complexity to the analysis process. In addition, as shown above and previously by others, it also makes the interpretation of the data more difficult and makes some steroids species very difficult to quantitate or even detect. The derivatisation of steroids also reduces the resolving power of comprehensive chromatography, as the functional groups are removed in the process. Effectively, it removes functional groups that potentially allow different hormones, which have very similar structures, to be resolved. The case for evaluating the elution characteristics of underivatised steroids by comprehensive chromatography is clear.

3.2.2. Chromatographic resolution of underivatised prohormones standards

A standard solution, containing a mixture of the twelve prohormones (833 ng/mL of each in acetonitrile), was prepared as described in Section 2.2.5. 1 μ L of the underivatised prohormone mixture (*i.e.* 833 pg of each injected on column) was analysed on the Leco Pegasus IV GCxGC-TOFMS in comprehensive, two-dimensional gas chromatography (2D-GC) mode, using the gas chromatography and mass spectrometry parameters summarised in Section 2.2.6. This quantity of steroids, approximately equivalent to 16.7 ng/mL in urine, was selected as the response factors of the steroids was at the time unknown. The column configuration incorporated a relatively non-polar J&W DB5-MS (30 m x 0.25 mm x 0.25

μm) column in the first dimension and a moderately polar BPX50 (2 m x 0.1 mm x 0.1 μm) in the second dimension, a configuration commonly used.

Before trying to understand the elution characteristic on both columns, it is appropriate to briefly review their chemical composition, as this will determine the partition coefficients of the analytes between the mobile and stationary phases and, in turn, why they were selected for this application. As shown in Figure 3.5, both DB5MS (BPX5 equivalent) and BPX50 have phenyl groups introduced in to the backbone (x) and as side chains (y) on to the dimethyl polysiloxane backbone of the stationary phase.

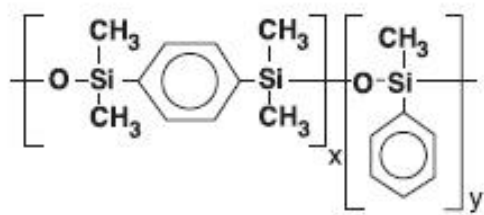


Figure 3.5. Substitution of the dimethyl polysiloxane backbone.

As the names suggest the proportion of the substitution is significantly greater in BPX50 (50%) than DB5MS (5%). This substitution helps make the stationary phase less likely to thermally degrade, thereby reducing column bleed, which is important when coupling to a mass spectrometer. The addition of these aromatic substituents slightly increases the polarity of the column and introduces aromaticity, which can interact with unsaturated bonds in the analytes. Given the chemical commonality in the substituents used, but different stoichiometry, one might anticipate that the elution characteristics should be similar in both columns. The significantly greater level of substitution in the BPX50 will amplify any interactions with unsaturated and polar groups in the analytes, in comparison to

DB5MS (BPX5), as will the smaller column diameter in the secondary column; however, it should also be remembered that the second column is significantly shorter and is held at a slightly higher temperature than the first column. The short retention time on the second column significantly limits which stationary phases can be utilised.

The first- and second- dimension retention times and the quantitation ion for each prohormone are summarised in Table 3.3. Before running the prohormone mixture the elution times of each of the prohormones were confirmed individually and their mass fragmentation patterns stored in a library.

Table 3.3. First- and second- dimension retention times obtained by GCxGC/TOFMS analysis of mixed standard of twelve prohormones, from a 1 μ L injection of a mixed standard containing 833 ng/mL of each steroid in acetonitrile.

Number	Prohormone	Quantitation Ion, m/z (Th)	First Dimension Retention Time (s)	Second Dimension Retention Time (s)
1	5(10)-estrene-3b,17b-diol	225	1214	2.430
2	4-estrene-3b,17b-diol	218	1226	2.435
3	5(10)-estrene-3,17-dione	131	1238	2.565
4	4-androstene-3b,17b-diol	220	1256	2.595
5	DHEA	255	1256	2.610
6	5(6)-androstene-3b,17b-diol	205	1268	2.655
7	5a-androstane-3,17-diol	292	1280	2.695
8	5a-androstane-3,17-dione	229	1286	2.810
9	Nandrolone	110	1304	2.905
10	5(6)-androstene-3,17-dione	124	1328	3.040
11	Testosterone	124	1346	3.110
12	1,4-androstadiene-3,17-dione	122	1352	3.185

The 2D surface plots, for the total ion chromatogram (TIC) obtained for the mixture of the twelve underivatised prohormones, are shown in Figure 3.6 and 3.7.

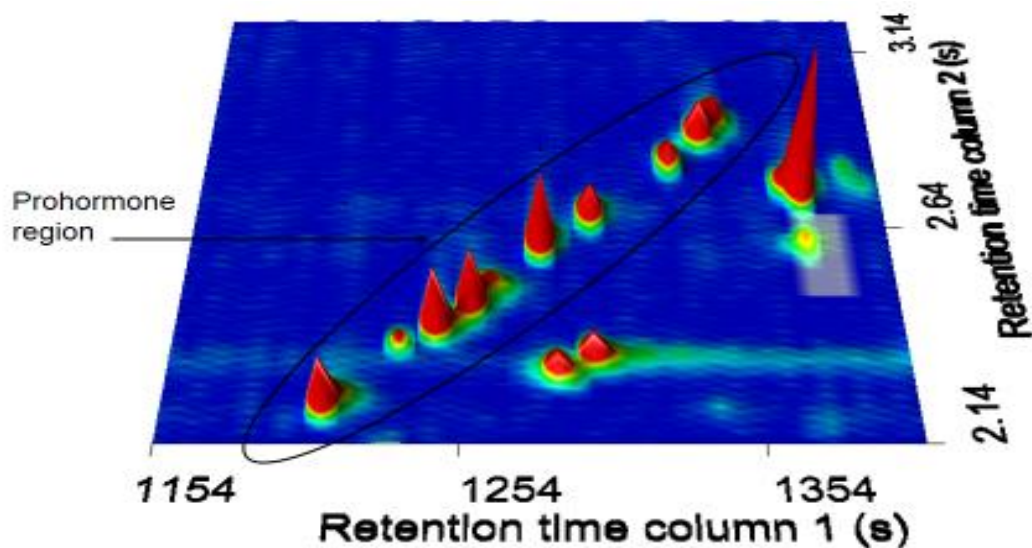


Figure 3.6. A 2D surface plot of the total ion chromatogram (TIC) illustrating the retention region of the underivatised prohormone species.

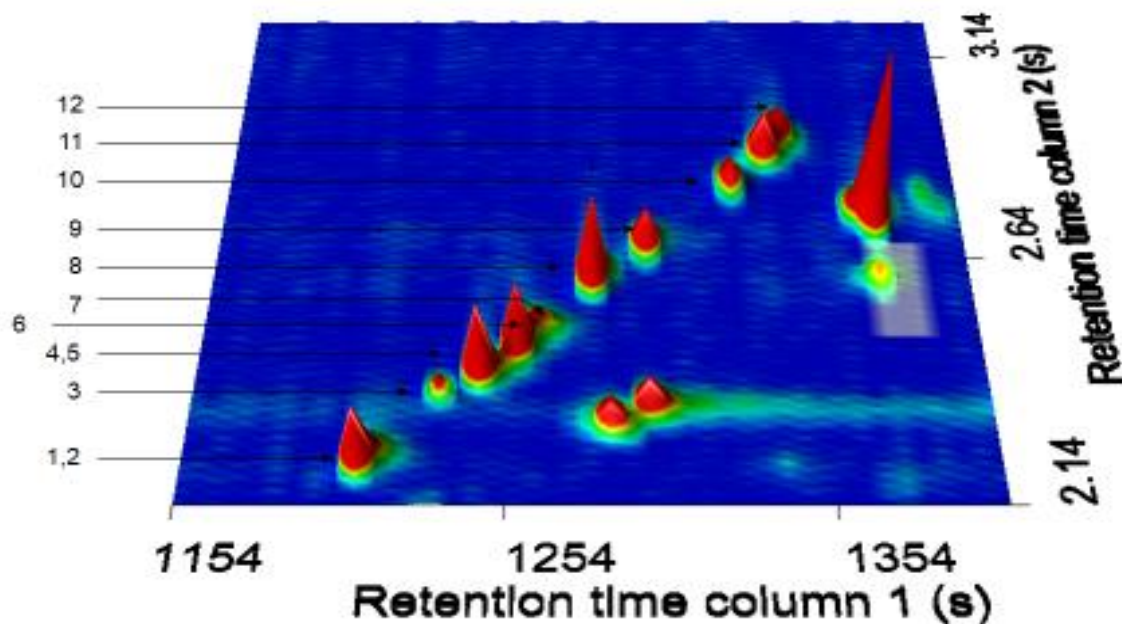


Figure 3.7. A 2D surface plot (TIC) illustrating the retention times of each of the underivatised prohormone species. A standard solution containing 833 pg of each of the twelve prohormones in acetonitrile was injected. 5(10)-estrene-3b,17b-diol [1] and 4-estrene-3b,17b-diol [2] co-elute, as do 4-androstene-3b,17b-diol [4] and DHEA [5].

Figure 3.6, clearly identifies the prohormone region; whereas, Figure 3.7 labels the location of each of the twelve prohormones. As well as resolving the prohormones in two dimensions it should be noted that this family of compounds are also well resolved from the column bleed and matrix compounds. In addition, as anticipated, the partitioning characteristics of the two columns are similar, with the result that the elution times of the analytes on the second column correlate with those of the first column, producing the distinctive shape of the prohormone region. On reviewing Figure 3.7, it became apparent that not all the prohormones were resolved in the total ion chromatogram. 5(10)-estrene-3b,17b-diol and 4-estrene-3b,17b-diol (Peaks #1 & #2) co-eluted; as did 4-androstene-3b,17b-diol and DHEA (Peaks #4 & #5). Through studying the individual fragmentation mechanisms of the prohormones, collated in Appendix A1.2, diagnostic ions for each of these compounds could be identified that enabled each of the twelve prohormones in the mixture to be uniquely identified and quantified.

As can be seen from these proposed mechanisms, the underivatised prohormones all follow similar, but subtly different, fragmentation processes. The range of fragmentation mechanisms are best illustrated by selecting one of the prohormones as a case study, in this case 4-androstene-3b,17b-diol. Here, electron ionization results in the formation of a molecular ion, at m/z 290, that readily eliminates water (18 Da) to form a fragment at m/z 272. This elimination means that the m/z 290 peak is significantly lower abundance than the m/z 272 fragment. The loss of ring A yields the base peak ion at m/z 220. In accordance with the fragmentation processes of most prohormones, the core backbone of the four rings remains intact, with the formation of the fragment ion at m/z 257, resulting from the elimination of a methyl radical (15 Da) from the m/z 272 fragment described earlier. The presence of a hydroxyl group in the C-17 position or C-3 position can result in elimination

of the water from both position as demonstrated in 5(10)-estrene-3b,17b-diol. The fragmentation profile provides important diagnostic value for the detection of prohormones in their native form. For example, the presence of the m/z 110 is highly indicative of the presence of nandrolone.

Figures 3.8 to 3.11 contain the 2D surface plots for these diagnostic ions and illustrate the power of coupling comprehensive chromatography with the high acquisition rate of the time of flight mass spectrometer.

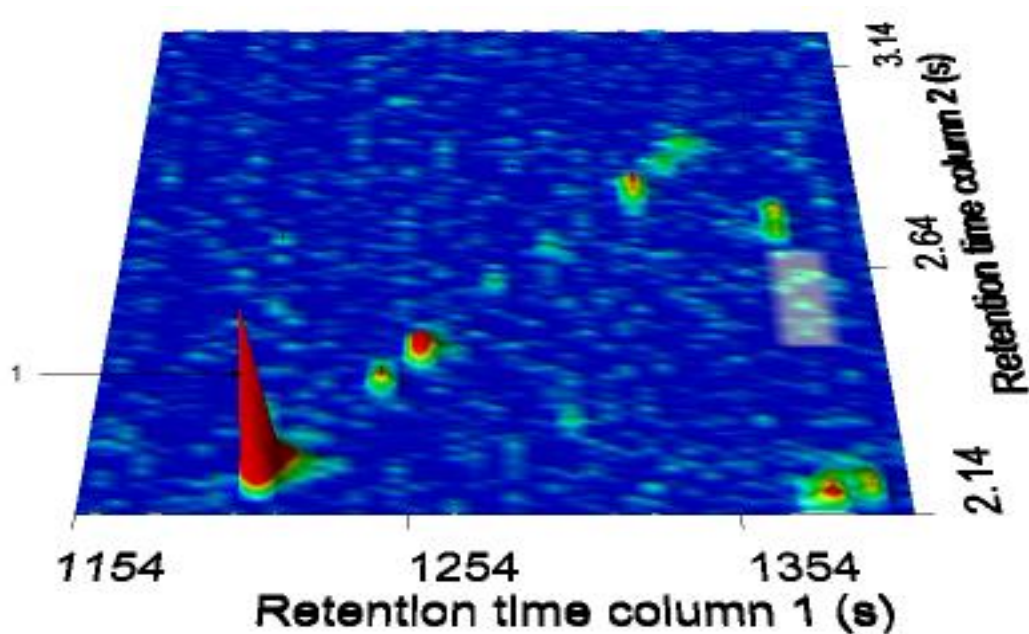


Figure 3.8. A 2D surface plot showing the location of 5(10)-estrene-3b,17b-diol using its diagnostic ion ($m/z = 225$).

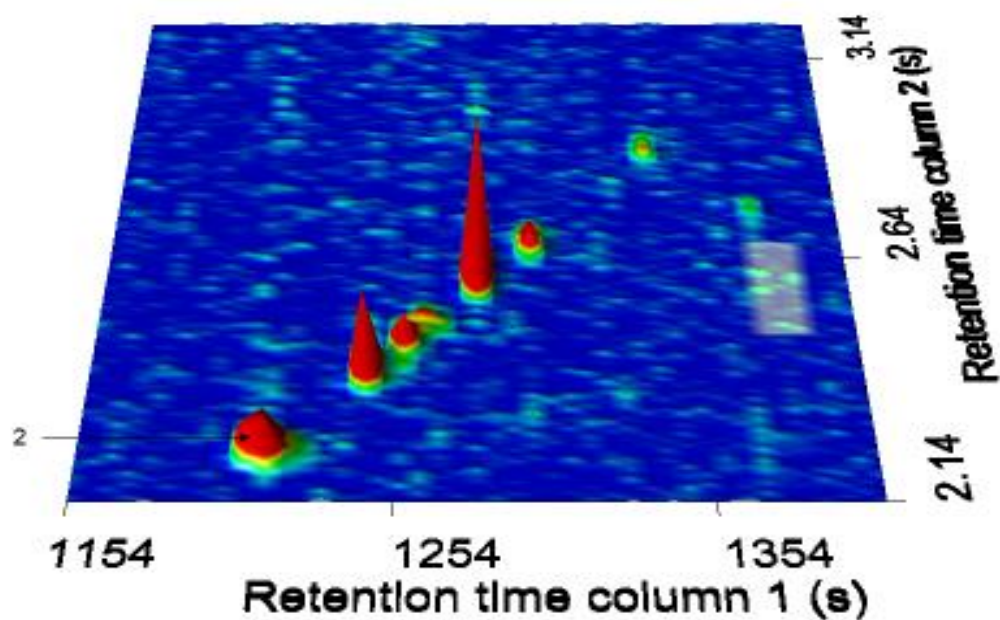


Figure 3.9. A 2D surface plot showing the location of 4-estrene-3b,17b-diol using its diagnostic ion ($m/z = 218$).

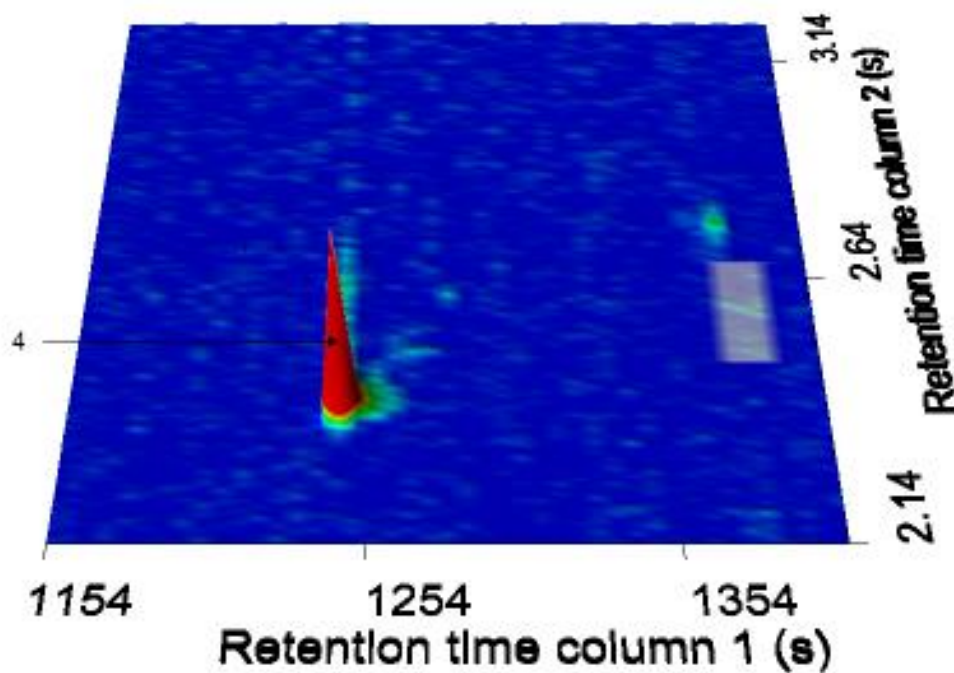


Figure 3.10. A 2D surface plot showing the location of 4-androstene-3b,17b-diol using its diagnostic ion ($m/z = 220$).

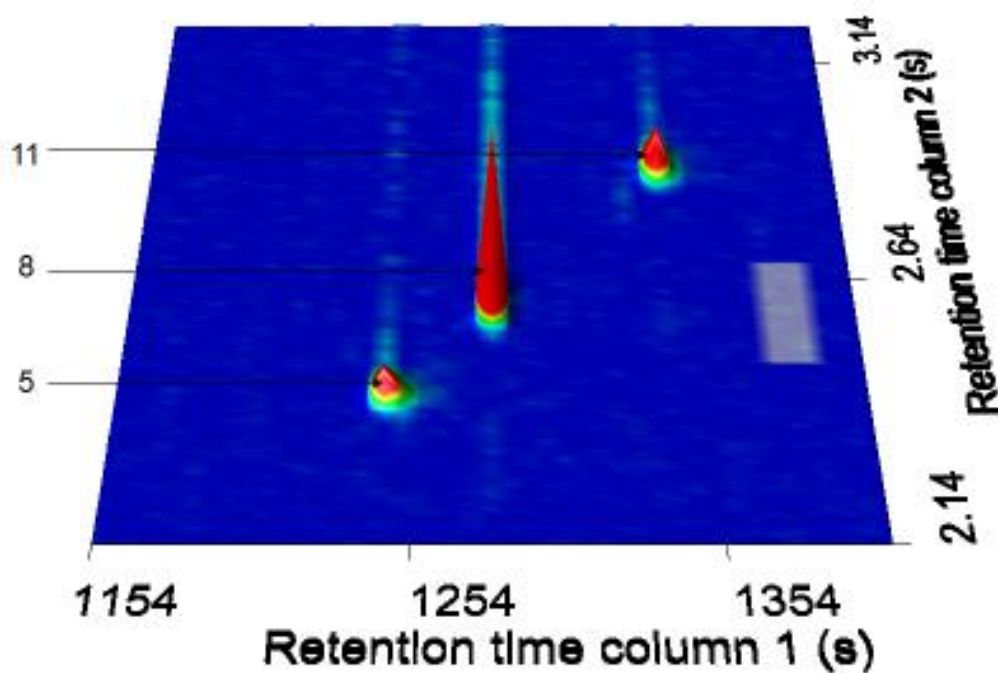


Figure 3.11. A 2D surface plot showing the locations of DHEA (#5), 5 α -androstane-3,17-dione [8] and testosterone [11], using the diagnostic ion ($m/z = 288$). Obtained from a 1 μ L injection of a standard solution containing the twelve prohormones at a concentration of 833 ng/ml of each, in acetonitrile.

By reviewing the chromatograms and the elution times in Table 3.3, some basic relationships can be derived from the interaction of the stationary phases with the prohormones. The diols elute between 1214 and 1280 s in the first dimension and between 2.430 and 2.695 s in the second. Similarly, the diones elute between 1238 and 1352 s in the first dimension and between 2.565 and 3.185 s in the second. DHEA (5(6)-androstene-3 β -ol-17-one), which has a carbonyl group at the 17-position and a hydroxyl group at the 3 β -position elutes at 1256 s / 2.610 s, respectively; whereas nandrolone (17 β -hydroxyestra-4-en-3-one) and testosterone (4-androstene-17-ol-3-one), which both have the carbonyl group at the 3-position, a C=C bond in the 4-position, as well as a hydroxyl group at the 17-position, elute at 1304s / 2.905 s and 1346 s / 3.110 s, respectively. The nor-19 steroids elute before the androsta(e)nes. Whilst there is overlap, generically we can state that diols

elute before steroids with both a carbonyl and hydroxyl group which, in turn, elute before diones.

As stated previously, the chemical composition of the two stationary phases are the same, except for the relative proportions of their substitution, therefore the mechanisms for partition of the steroids between the carrier gas and the stationary phase of the first column will be similar to that with the secondary column. The partition coefficients between the mobile and stationary phase will be driven by a combination of the interactions *e.g.*, dispersion (non-polar components), dipole-dipole (carbonyl and hydroxyl groups), π - π (unsaturated groups) with i) the dimethyl polysiloxane segment of the stationary phase and ii) the phenyl substituents. The different shapes/configurations of the prohormones will also influence how the various function groups interact with the stationary phase. In the first column, with only 5 % substitution, interactions with the dimethyl portion is likely to dominate, even though dispersion forces are much weaker than the others; whereas, in the secondary column, with 50 % substitution, the interactions with the phenyl substituents will be much more significant. The much smaller diameter of the secondary column will also significantly facilitate interactions with the stationary phase, allowing much shorter columns to achieve compound separation within the modulation time.

The partition coefficient will be driven by subtle differences in: the overall shape of the sample molecules; the nature, number and location of functional groups present; the number and location of the double bonds in the rings. The three-dimensional conformation of the parent 17-carbon tetracyclic will be strongly influenced by the location of an unsaturated carbon-carbon bond and the presence of a carbonyl group (C=O) in place of a hydroxyl group. The presence of the double bond will result in the carbon atoms adopting a planar (120° bond angle, sp^2 hybridisation) arrangement instead of the tetrahedral

arrangement (109.5° angle, sp^3 hybridisation) when only single bonds are present. Double bonds are also shorter than single bonds, which will result in a slight twisting of the cyclohexane ring. It appears that the presence of C=C and C=O bonds both enhance interactions with the stationary phase and result in their partitioning on to the mobile phase, presumably as a result of interactions with the aromatic phenyl groups.

It is likely that the separation in the first column is mainly driven by the relative volatility of the species present. Indeed, apart from nandrolone, the three 19-norsteroids, lacking a methyl group attached to the 10-position, elute first in both the first and second dimension

To summarise the findings:

- i. 19-norsteroids elute before their androsta(e)ne equivalents (as evidenced by 4-estrene-3b,17b-diol eluting before 4-androstene-3b,17b-diol and nandrolone eluting before testosterone);
- ii. Diols elute before diones (evidenced by the fact that 5 of the first seven species are diols and 3 of the last five species are diones);
- iii. The presence of a carbonyl group at the 3- position as well as double bond at the 4- position of Ring A (nandrolone and testosterone) increases retention on the columns, even with a hydroxyl group at the 17-position, this is likely to be due to delocalisation of the charge over between the double bonds and its interaction with the aromatic substituents in the stationary phase;
- iv. Nor-19 steroids, separated only by the location of a double bond (as evidenced by 5(10)-estrene-3b,17b-diol and 4-estrene-3b,17b-diol) will effectively co-elute, but may be distinguished by their diagnostic fragmentation ions;

- v. Androstenes, separated only by the location of a double bond can be resolved (as evidenced by the separation of 5(6)-androstene-3 β ,17 β -diol and 4-androstene-3 β ,17 β -diol);
- vi. In terms of diones, the elution order is estrene, androstane, androstene and androstadiene.

3.2.3. Validation studies for the use of GCxGC/MS with underivatised prohormone standards

As described in Section 2.2.7, the mixed standard, in acetonitrile, was used to prepare a 5-point calibration curve at concentrations of 500, 200, 150, 125 and 50 ng/mL for each steroid. Based on a 1 μ L splitless injection, this is equivalent to between 500 - 50 pg of each steroid being injected on column. If one also takes in to consideration the MIP method developed and described in Section 2.2.9, and assuming complete recovery, the quantity of prohormone injected would be equivalent to that extracted from urine samples containing between 1 and 10 ng/mL (WADA MRPL 2010) of each steroid, respectively. The equations and squared correlation coefficients for the linear least square fit for the peak area for each of the steroids is summarised in Table 3.4 and the plots for each of the steroids are provided in Figure A2 in Appendix A2. The ions used for quantitation were listed in Table 3.3. All prohormones were found to give a linear signal over the concentration range measured.

Table 3.4. The equations and the squared correlation coefficients for the linear least square fit for each of the steroids over the range of 50-500 pg injected

Number	Prohormone	Regression Equation	R ²
1	5(10)-estrene-3b,17b-diol	$y = 32.12 x - 871.77$	0.9949
2	4-estrene-3b,17b-diol	$y = 37.29 x - 1379.30$	0.9980
3	5(10)-estrene-3,17-dione	$y = 44.28 x - 154.39$	0.9954
4	4-androstene-3b,17b-diol	$y = 158.98 x - 5041.39$	0.9959
5	DHEA	$y = 57.76 x - 1295.39$	0.9980
6	5(6)-androstene-3b,17b-diol	$y = 20.05 x - 654.69$	0.9952
7	5a-androstane-3,17-diol	$y = 49.60 x - 2727.22$	0.9957
8	5a-androstane-3,17-dione	$y = 89.59 x + 1301.20$	0.9939
9	Nandrolone	$y = 233.03 x - 5011.66$	0.9975
10	5(6)-androstene-3,17-dione	$y = 152.22 x - 234.54$	0.9956
11	Testosterone	$y = 628.05 x - 46215.40$	0.9842
12	1,4-androstadiene-3,17-dione	$y = 1273.07 x - 5398.49$	0.9989

As reported in Section 2.2.7, the theoretical limits of detection (LOD) and quantitation (LOQ) for each prohormone were estimated by repeat injections (x10) of 1 µL of the lowest concentration standard (50 ng/mL), equivalent to 50 pg on column. The limit of detection for each was defined as 3 times the standard deviation from the average obtained; whereas the limit of quantification was taken as being 10 times the standard deviation. The standard deviation is calculated by multiplying the relative standard deviation of the peak areas measured for the quantitation ion by the mass injected (50 pg), as summarised in Table 3.5.

Table 3.5. Theoretical limit of detection and quantitation calculated for each prohormone

Number	Prohormone	Peak Area (RSD)	LOD (pg)	LOQ (pg)
1	5(10)-estrene-3b,17b-diol	0.131	19.7	65.5
2	4-estrene-3b,17b-diol	0.092	13.8	46.0
3	5(10)-estrene-3,17-dione	0.137	20.6	68.5
4	4-androstene-3b,17b-diol	0.180	27.0	90.0
5	DHEA	0.122	18.3	61.0
6	5(6)-androstene-3b,17b-diol	0.175	26.3	87.5
7	5a-androstane-3,17-diol	0.078	11.7	39.0
8	5a-androstane-3,17-dione	0.121	18.2	60.5
9	Nandrolone	0.150	22.5	75.0
10	5(6)-androstene-3,17-dione	0.086	12.9	43.0
11	Testosterone	0.168	25.2	84.0
12	1,4-androstadiene-3,17-dione	0.102	15.3	51.0

Based on the results obtained from this standard method for the estimation of the limits of detection and quantitation, it would appear that the twelve prohormones should all be detectable below ~30 pg on column; and quantitation should be possible below ~100 pg on column.

In terms of sensitivity, this method would indicate that 4-androstene-3b,17b-diol will be the hardest to detect and quantify, followed by 5(6)-androstene-3b,17b-diol and testosterone. Conversely, 5a-androstane-3,17-diol, 4-estrene-3b,17b-diol and 5(6)-androstene-3,17-dione should be detectable at lower levels. As these figures are estimates, based on the reproducibility of the peak area measurements, it remains to be determined if these levels are achievable, in particular when extracted from urine.

The LOD and LOQ values reported are based on a 1 μL injection from prohormone standards in acetonitrile at a concentration of 50 ng/mL, i.e. 50 pg injected on column. Based on a proposed concentration factor of at least 50, for the extraction of urine samples (1 mL of urine onto MIPs followed by elution into 1 mL of methanol, drying and reconstituting into 20 μL of acetonitrile) and the injection of 1 μL of this reconstituted solution, one might expect that the method developed during this study should be capable of detecting the prohormones at, or below, 1 ng/mL level in urine samples. Levels that are well below both of the latest WADA minimum required performance level (MRPL) values for most androgenic anabolic steroids in urine (10 and 5 ng/mL, respectively).

There is, of course, the caveat that most steroids are present in urine in the conjugated form and would probably require hydrolysis prior to MIPs extraction. As previously reported unconjugated steroids make up approximately ~3% of the total steroids present in urine. At the 2010 WADA levels, this would have necessitated a limit of detection of ~15 pg on column. Interestingly, most of the prohormones in Table 3.5 are estimated to be detectable at around that level.

Therefore, with the caveat that a hydrolysis method will probably be required to deconjugate the steroids, prior to MIPs analysis, the method was determined to be fit for its intended purpose of detecting androgenic anabolic steroids in urine samples at the WADA limits of 10 ng/mL and should be explored further. With MRPL value now lowered to 5 ng/mL, for most species in 2012, and assuming that the unconjugated steroids are present at 3% of all steroids in a urine sample, then the current method would not be directly applicable to untreated urine samples. This would require a limit of detection of ~ 8 pg on column ($5/33 = 0.15$ ng/mL in urine). It should, however, be noted that the level of detection in this study could be further improved by either: increasing the quantity of urine

passed over a larger MIPs cartridge or by utilising a greater proportion of the sample extracted. For example, by increasing the volume (from 1 μL up to 20 μL) of acetonitrile injected in to the mass spectrometer, through the use of a programmable temperature vapourisation (PTV) injector such as the Optic 4 from ATAS GL, or similar devices from Agilent Technologies or Gerstel. This will require further investigation, as the ability to directly extract a family of unconjugated steroids from urine without prior treatment will be a significant step forward.

Whether such approaches work will depend on the ability of the MIPs to resolve the target compounds from the matrix peaks that will be present if larger volumes of urine or solvent are used and ultimately the background noise for the quantitation ion in the chromatographic region of interest.

3.2.4. Evaluating the performance of molecularly imprinted polymers for the extraction of underivatised prohormones from spiked synthetic urine.

The molecular imprinted polymers (M5 & M6), templated with testosterone, and the non-imprinted polymers (B5 & B6) were prepared as described in Section 2.2.8. Prior to use, following crushing in a mortar and pestle, wet-sieving through a mesh and collection of the 38 to 63 μm fraction, the polymers produced were imaged on a scanning electron microscope (SEM).

As can be seen in Figures 3.12 and 3.13, the MIPs particles are irregular in shape and highly angular, which would suggest that further crushing in the mortar and pestle would be required to make them more spherical, which would be more suitable for the ideal packing of a solid phase extraction (SPE) tube. From the images collected the sizes of the particles appear to be similar with the dimensions of the individual particles being consistent with the mesh size of the sieves used. Whether further crushing is necessary is debatable since the generation of more spherical particles would inevitably result in smaller particles, which could restrict the flow of the solvent through the SPE cartridge. Conversely, highly irregular and angular particles could result in open channels or pathways through the SPE cartridges which could prevent the target species sufficiently interacting with the MIPS, resulting in them being carried straight through. The highly labour intensive and time-consuming nature of the crushing and wet-sieving process, as used in this study, also puts constraints on what is viable. Automation of the process is feasible and would be necessary for this technique to be implemented on a wider scale.

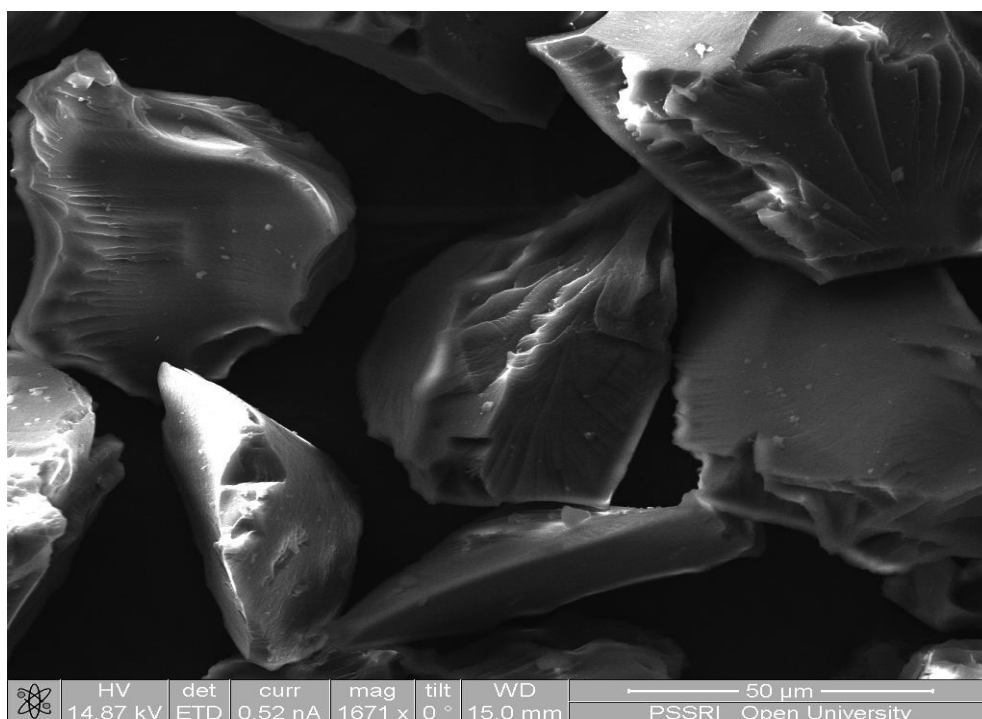


Figure 3.12. A close up image (1671x magnification) of the M5 molecular imprinted polymers obtained using a scanning electron microscope.

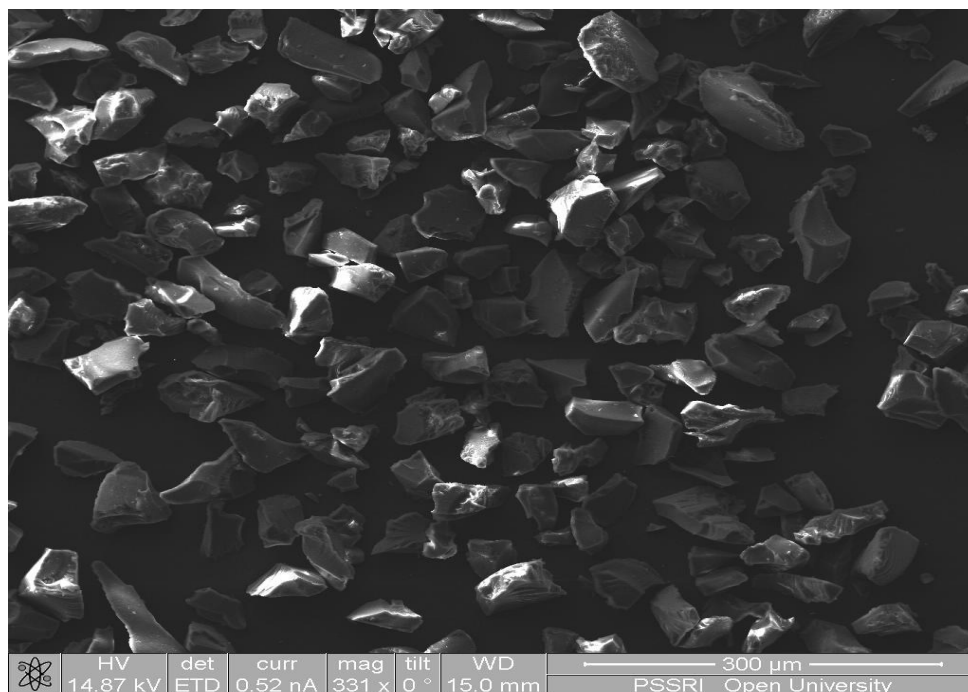


Figure 3.13. An image (331 x magnification) of the M6 molecular imprinted polymers obtained using a scanning electron microscope.

The percentage recoveries of each of the target analytes on triplicate cartridges containing either imprinted (M5/M6) or non-imprinted (B5/B6) polymers were determined using the method specified in Section 2.2.9. In brief: 1 mL of synthetic urine was spiked at the 10 ng/mL level for each prohormone; it was then added to each of the MIPs cartridges, washed and then eluted. The eluents were then dried and reconstituted in acetonitrile and the hormones were injected underivatized in the comprehensive gas chromatography system. Before we study the performance of the polymers, it is worth reviewing the qualitative evidence provided by these chromatograms, of the additional benefits of utilising molecularly imprinted polymers, and also the quality of the chromatograms obtained.

One of the proposed benefits is that they can be highly specific and significantly reduce the level of non-specific binding between the stationary phase and matrix compounds. This should result in a significant reduction in the quantity of matrix compounds that are introduced into the GC-MS or GCxGC-MS. In traditional single dimension GC-MS analyses, these matrix species would co-elute with target analytes, interfering with their quantitative determination. In comprehensive chromatography, as shown in Figure 3.15, this is less of an issue; however, in terms of long term robustness of the method, a reduction in the level of non-target species being introduced is going to be advantageous, as it reduces the need to clean the injector, the column and the mass spectrometer ion source.

Figure 3.14 illustrates the level of matrix compounds that are present when a spiked 1 mL synthetic urine sample is extracted with a) C18 (Strata) SPE cartridge, b) M5 MIPs imprinted with testosterone and using methacrylic acid (MAA) as the monomer, and c) M6 MIPs imprinted with testosterone and using 4-vinyl pyridine (4-VP) as the monomer.

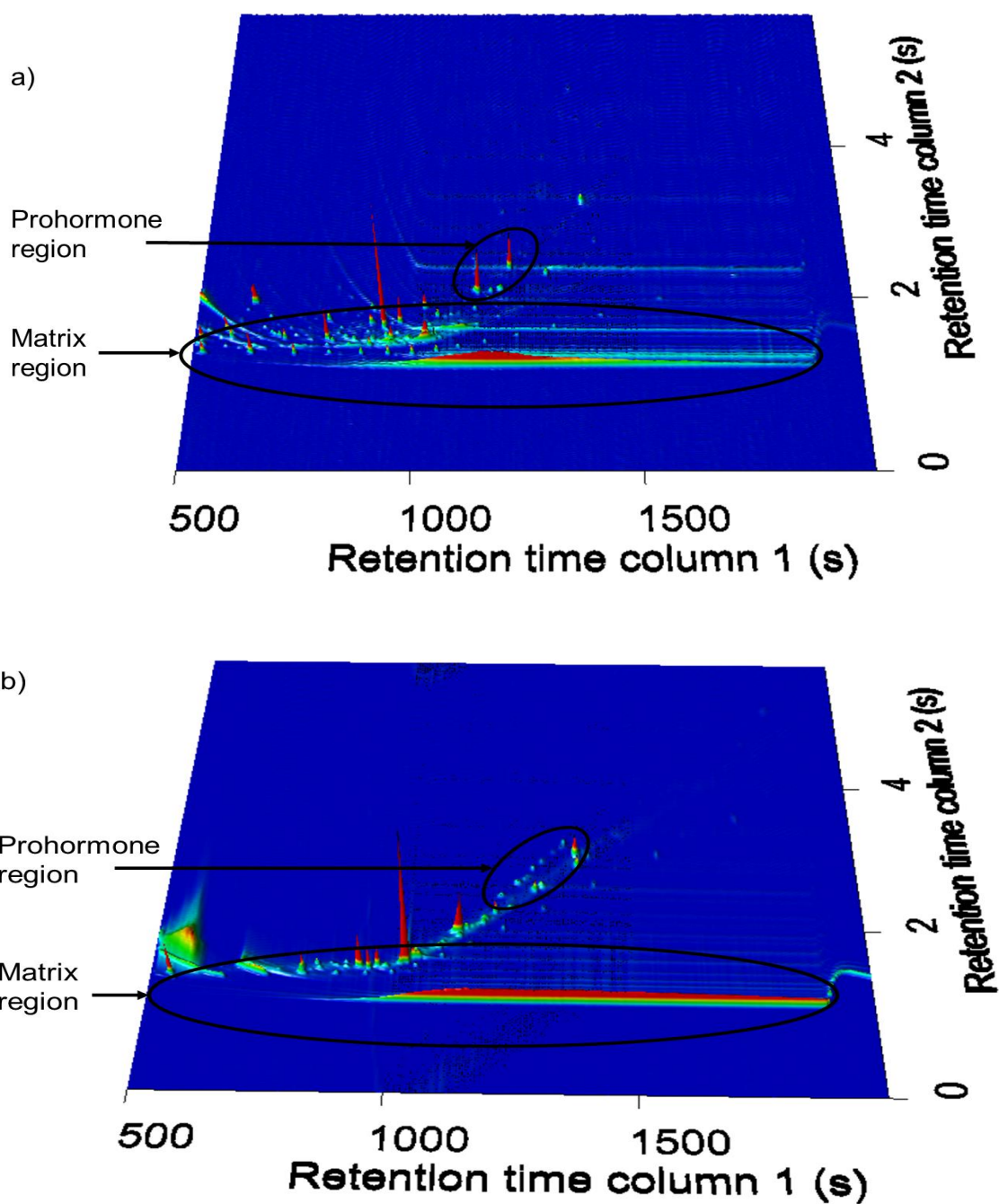


Figure 3.14. 2D surface plots (TIC), showing the extent of the matrix present in the eluents from 1 mL of synthetic urine spiked at the 10 ng/mL level per prohormone: a) C18 (Strata) SPE cartridge, b) M5 (MIPS cartridge) with MAA as the monomer.

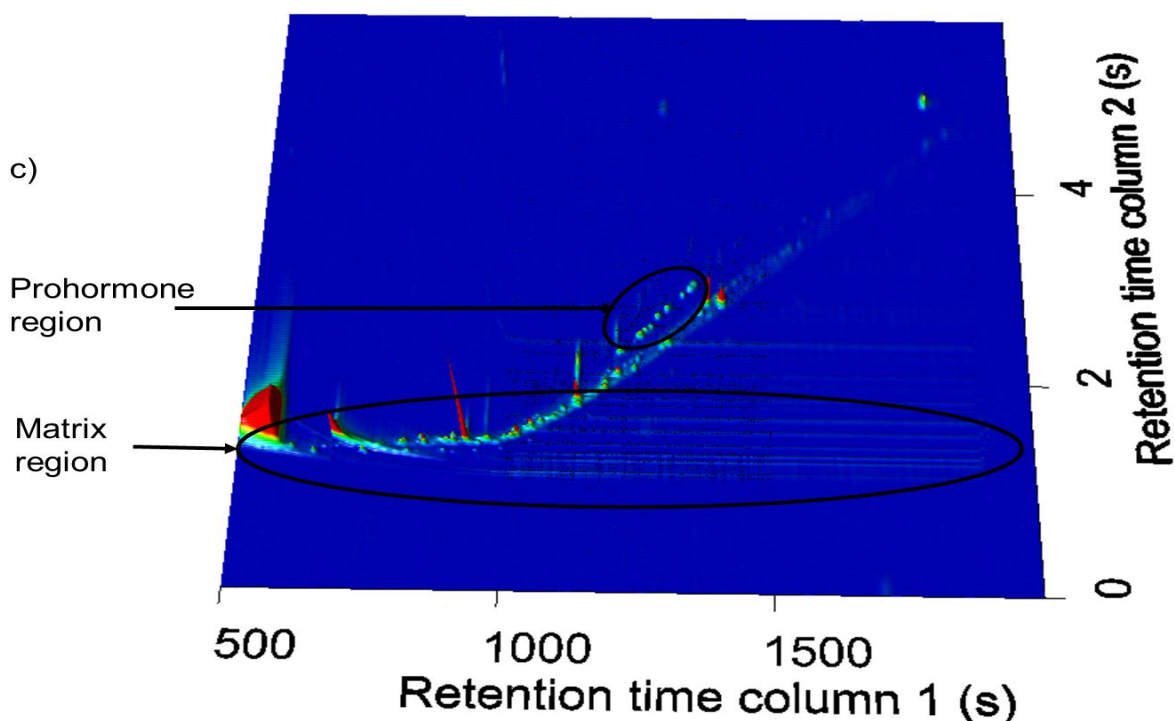


Figure 3.14 2D surface plots (TIC), showing the extent of the matrix present in the eluents from 1 mL of synthetic urine spiked at the 10 ng/mL level per prohormone:
c) M6 (MIPS cartridge) with 4-VP as the monomer

The 2D surface plots would seem to indicate that the eluent from the M6 cartridge has significantly lower levels of matrix species than M5 which, in turn, has much less than the SPE cartridge. This would suggest that the use of 4-vinyl pyridine as the monomer in M6 is resulting in a reduction of the non-specific binding relative to the other two. As it appears to also extract the prohormones, this may imply the development of specific cavities templated by testosterone. The corresponding 2D surface plots for the non-imprinted polymers are shown in Figure 3.15. Interestingly, the level of matrix present for B5 does not appear to be as great as for M5. This suggests that following templating, the MAA monomer is resulting in both specific and non-specific binding of matrix compounds, as well as the target species.

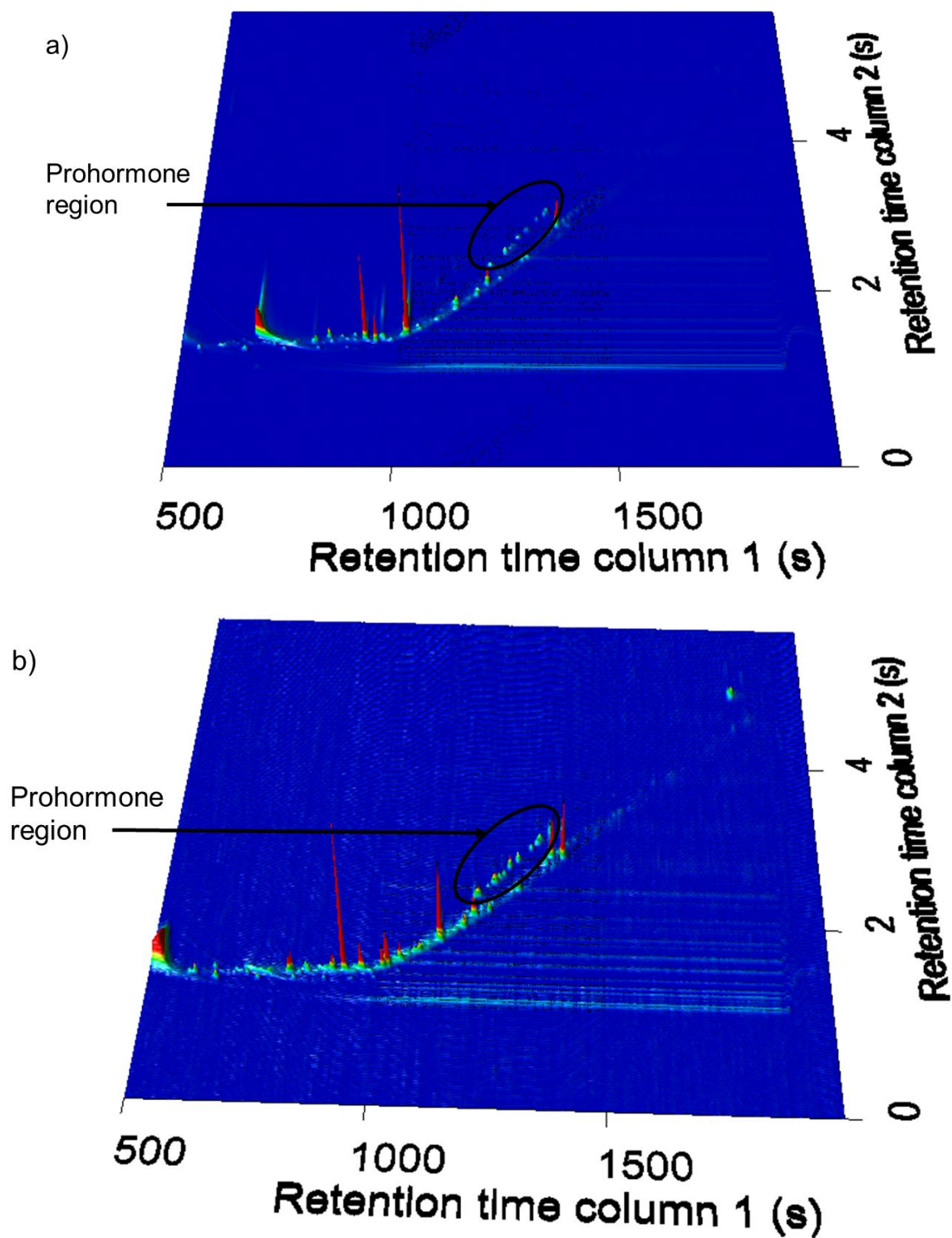


Figure 3.15. 2D surface plots (TIC), showing the extent of the matrix present in the eluents from 1 mL of synthetic urine spiked at the 10 ng/mL level per prohormone:

- a) B5 (non-imprinted cartridge) with MAA as the monomer
- b) B6 (non-imprinted cartridge) with 4-VP as the monomer

Having explored the influence of the polymers based on the specificity of the matrix species extracted, it is appropriate to review the chromatograms obtained for the prohormones. Figure 3.16 shows a close up of the prohormone region for the eluent from the M5 imprinted cartridge. As stated previously, this 2D surface plot of the total ion current (TIC) has resulted from the spiking of a 1 mL of synthetic urine sample at 10 ng/mL and the processing of the sample using the method described in Section 2.2.9.

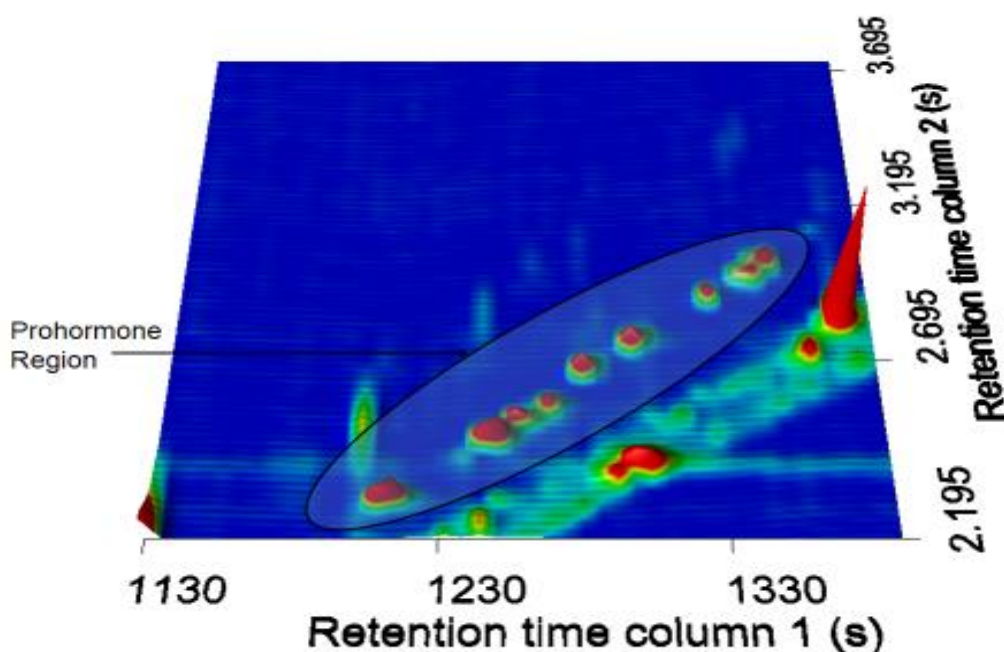


Figure 3.16. A 2D surface plot (TIC) showing the prohormone region for the eluent of the M5 imprinted cartridge, following extraction from 1mL of spiked synthetic urine.

All of the 12 prohormones are detected and elute in the same order as in the standard samples shown previously. Figure 3.17 labels each of the prohormones in the TIC, whereas Figure 3.18 illustrates how 5(10)-estrene-3-17-dione can be better identified and quantified by selecting one of its diagnostic fragmentation ions ($m/z = 131$).

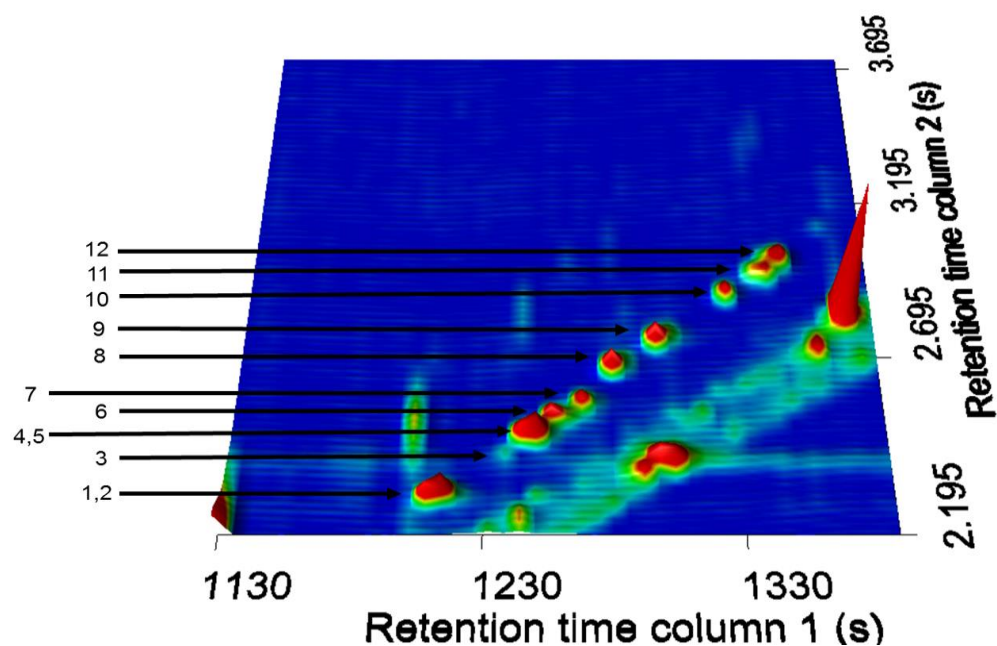


Figure 3.17. A 2D surface plot (TIC) showing the positions of each of the prohormones for the eluent of the M5 imprinted cartridge, following extraction from spiked synthetic urine.

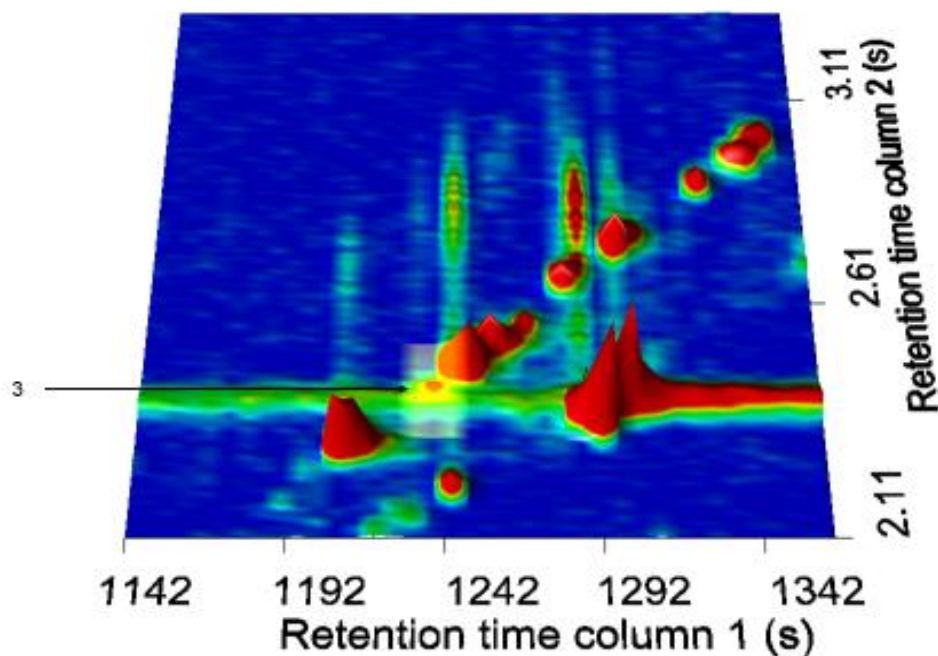


Figure 3.18. A 2D surface plot showing how 5(10)-estrene-3-17-dione, can be better characterised by extracting its diagnostic ion ($m/z = 131$). The sample results from extraction by M5 of 1mL of synthetic urine spiked at 10 ng/mL.

Figure 3.19 identifies each of the prohormones seen in a 2D surface plot of the total ion current in the eluent of M6 imprinted polymer extraction of the spiked synthetic urine. Whilst, Figures 3.20 and 3.21 illustrate how 5(10)-estrene-3b,17b-diol and 4-estrene-3b,17b-diol can be resolved using their diagnostic ions, $m/z = 225$ and 210 , respectively.

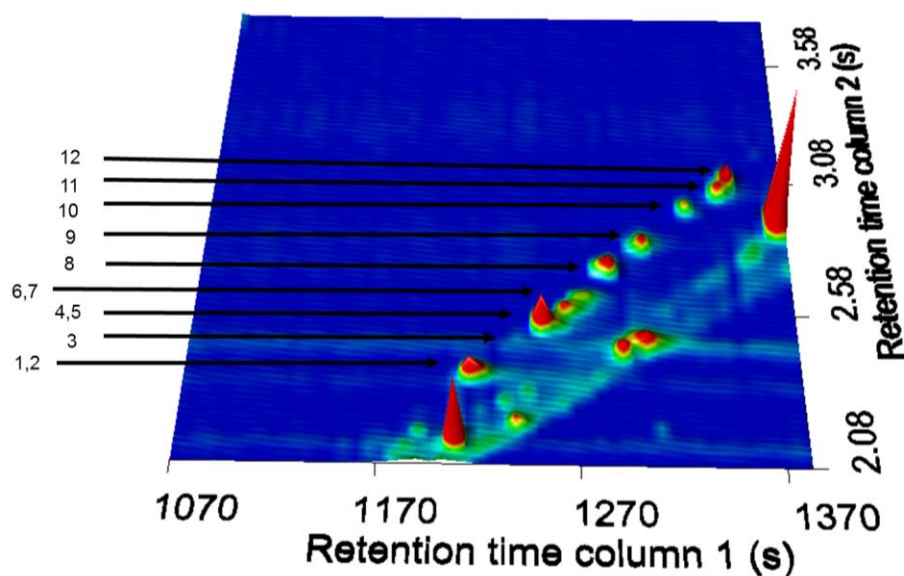


Figure 3.19. A 2D surface plot (TIC) showing positions of each of the prohormones for M6 MIPs extraction of 1 mL of synthetic urine spiked at 10 ng/mL for each. 5(6)-androstene-3b,17b-diol [6] and 5a-androstane-3,17-diol [7] are resolved but hard to label.

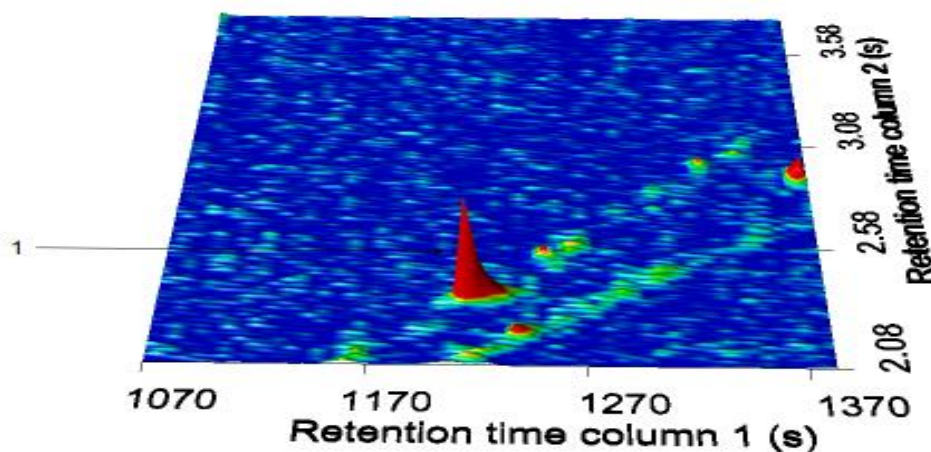


Figure 3.20. A 2D surface plot showing the location of 5(10)-estrene-3b,17b-diol using its diagnostic ion ($m/z = 225$).

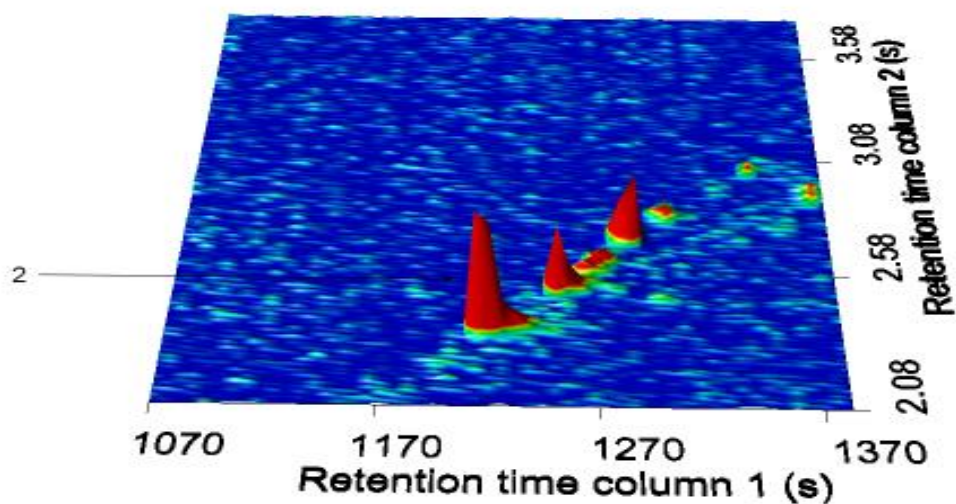


Figure 3.21. A 2D surface plot showing the location of 4-estrene-3b,17b-diol to the bottom left, using its diagnostic ion ($m/z = 218$).

Figure 3.22 provides an alternative perspective for the same deconvolution of 5(10)-estrene-3b,17b-diol [1] and 4-estrene-3b,17b-diol [2], using their diagnostic ions.

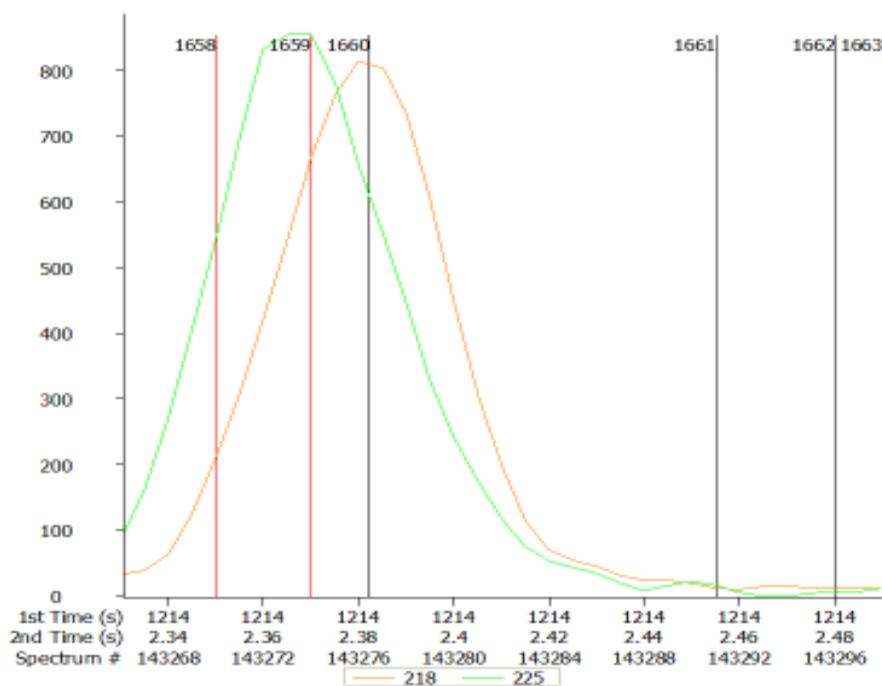


Figure 3.22. 2D raw data showing the elution characteristics of $m/z = 225$ and $m/z = 218$ ions in both chromatographic dimensions, along with the spectrum numbers and peak markers.

Applying the same rationale, Figures 3.23 to 3.25 illustrate how 4-androstene-3 β ,17 β -diol [4] and DHEA [5] can be resolved using the retention times of their diagnostic fragmentation ions.

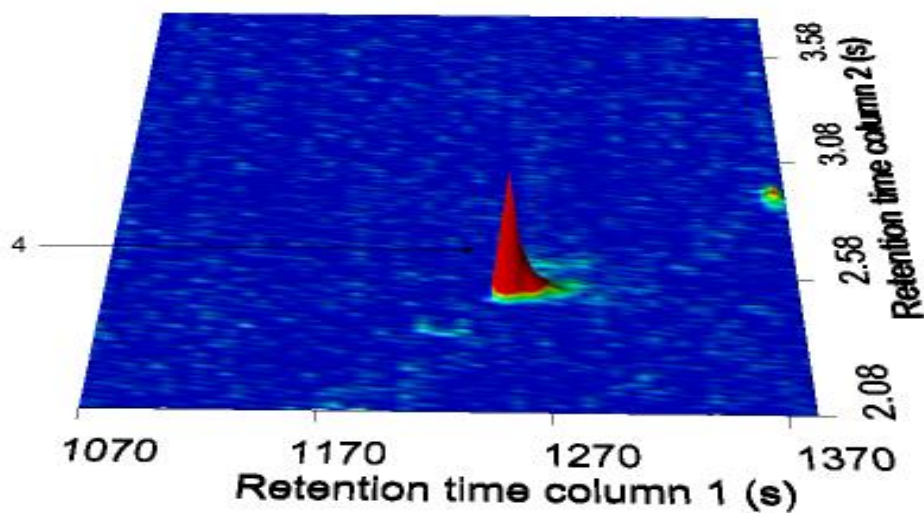


Figure 3.23. A 2D surface plot showing the location of 4-androstene-3 β ,17 β -diol using its diagnostic ion ($m/z = 220$).

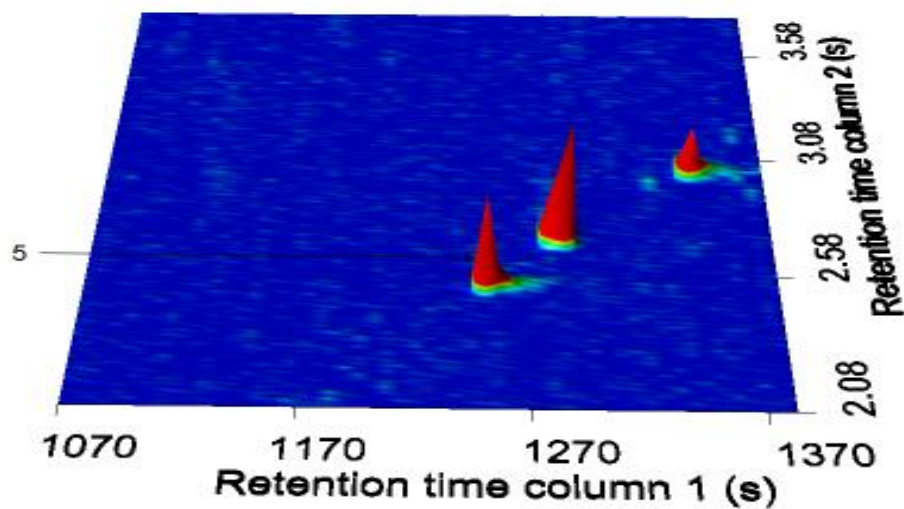


Figure 3.24. A 2D surface plot showing the location of DHEA, using its diagnostic ion ($m/z = 288$).

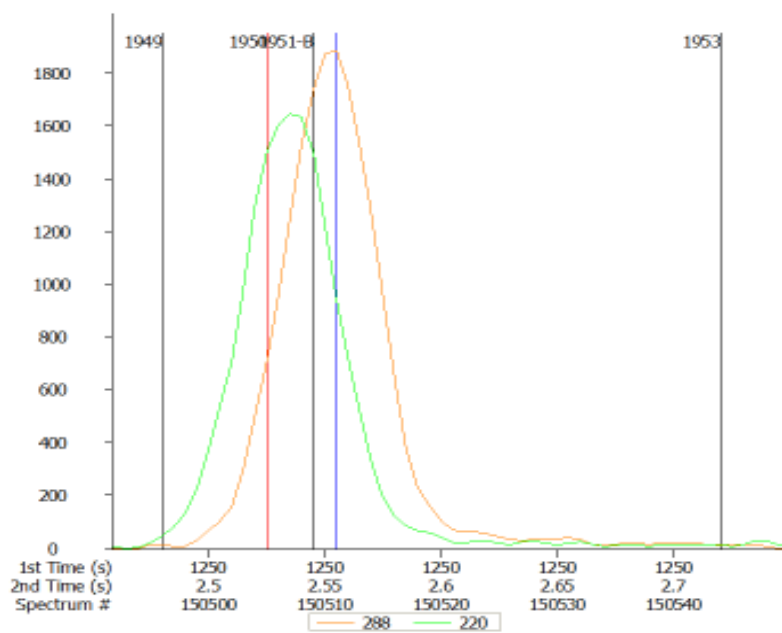


Figure 3.25. 2D raw data showing the elution characteristics of $m/z=288$ and $m/z=220$ ions in both chromatographic dimensions, along with the spectrum numbers and peak markers.

3.2.5. Evaluating the percentage recovery of the prohormones from synthetic urine using cartridges containing imprinted (M5) and non-imprinted (B5) polymers that use MAA as the monomer

Based on the method described in Section 2.2.9, the performance of imprinted and non-imprinted polymers, utilising MAA as the monomer, were evaluated. The results obtained for each prohormone are recorded in Tables 3.6 (M5, imprinted) and 3.7 (B5, non-imprinted), respectively. Table 3.8 then evaluates whether imprinting with testosterone as the template had a significant influence on the extraction of the prohormones from urine.

Table 3.6. Percentage recoveries of each of the prohormones, based on their extraction from 1 mL of synthetic urine (spiked at 10 ng/mL) added to three cartridges of the imprinted MIPs (M5).

Number	Prohormone	Recovery M5.1 (%)	Recovery M5.2 (%)	Recovery M5.3 (%)	Average Recovery M5 (%)	Standard Deviation σ_{n-1} (%)
1	5(10)-estrene-3b,17b-diol	86.2	84.0	79.4	83.2	3.5
2	4-estrene-3b,17b-diol	84.8	77.3	79.2	80.4	3.9
3	5(10)-estrene-3,17-dione	99.3	100.7	113.3	104.4	7.7
4	4-androstene-3b,17b-diol	105.4	110.9	98.7	105.0	6.1
5	DHEA	73.3	76.0	74.2	74.5	1.4
6	5(6)-androstene-3b,17b-diol	86.7	77.1	66.9	76.9	9.9
7	5a-androstane-3,17-diol	59.9	60.0	75.6	65.2	9.1
8	5a-androstane-3,17-dione	86.9	88.8	76.8	84.1	6.4
9	Nandrolone	76.8	82.7	69.9	76.5	6.4
10	5(6)-androstene-3,17-dione	80.2	79.6	69.8	76.5	5.8
11	Testosterone	79.9	86.1	76.4	80.8	4.9
12	1,4-androstadiene-3,17-dione	89.7	94.2	85.4	89.8	4.9

Table 3.7. Percentage recoveries of each of the prohormones, based on their extraction from 1 mL of synthetic urine (spiked at 10 ng/mL) added to three cartridges of the non-imprinted polymer (B5).

Number	Prohormone	Recovery B5.1 (%)	Recovery B5.2 (%)	Recovery B5.3 (%)	Average Recovery B5 (%)	Standard Deviation σ_{n-1} (%)
1	5(10)-estrene-3b,17b-diol	44.9	56.1	60.5	53.8	8.1
2	4-estrene-3b,17b-diol	53.8	81.1	73.4	69.4	14.1
3	5(10)-estrene-3,17-dione	57.7	77.5	88.2	74.5	15.5
4	4-androstene-3b,17b-diol	78.5	115.4	113.5	102.5	20.8
5	DHEA	70.6	74.4	72.1	72.4	1.9
6	5(6)-androstene-3b,17b-diol	61.5	83.1	77.3	74.0	11.2
7	5a-androstane-3,17-diol	60.2	71.4	77.3	69.7	8.7
8	5a-androstane-3,17-dione	66.0	87.1	83.2	78.8	11.3
9	Nandrolone	63.5	79.3	78.5	73.8	8.9
10	5(6)-androstene-3,17-dione	75.1	79.8	80.5	78.5	3.0
11	Testosterone	63.4	82.6	79.4	75.1	10.3
12	1,4-androstadiene-3,17-dione	83.4	102.5	100.5	95.5	10.5

Table 3.8. An evaluation of whether the imprinting with testosterone had a significant influence on the extraction of the prohormones from urine.

Number	Prohormone	Average Recovery M5 (%)	Average Recovery B5 (%)	Significant Influence at the 2 σ level?
1	5(10)-estrene-3b,17b-diol	83.2 +/- 6.9	53.8 +/- 16.1	Yes
2	4-estrene-3b,17b-diol	80.4 +/- 7.8	69.4 +/- 28.2	No
3	5(10)-estrene-3,17-dione	104.4+/-15.4	74.5+/- 31.0	No
4	4-androstene-3b,17b-diol	105.0 +/- 12.2	102.5 +/- 41.6	No
5	DHEA	74.5 +/- 2.8	72.4 +/- 3.8	No
6	5(6)-androstene-3b,17b-diol	76.9 +/- 19.8	74.0 +/- 22.3	No
7	5a-androstane-3,17-diol	65.2 +/- 18.1	69.7+/- 17.4	No
8	5a-androstane-3,17-dione	84.1 +/- 12.8	78.8 +/- 22.5	No
9	Nandrolone	76.5 +/- 12.8	73.8 +/- 17.8	No
10	5(6)-androstene-3,17-dione	76.5 +/- 11.6	78.5 +/- 5.9	No
11	Testosterone	80.8 +/- 9.8	75.1 +/- 20.6	No
12	1,4-androstadiene-3,17-dione	89.8 +/- 8.8	95.5 +/- 21.0	No

As can be seen in Table 3.6, the percentage recovery of the 12 prohormones, using the M5 imprinted polymer, ranged between 65 and 105 % for the diols; whereas the recovery of DHEA, nandrolone and testosterone, respectively, ranged from 75 to 81 %. The diones had a percentage recovery of between 77 and 105 %.

The percentage recovery of the 12 prohormones, using the B5 non-imprinted polymer, ranged between 54 and 102 % for the diols; whereas the recovery of DHEA, nandrolone and testosterone, respectively, ranged from 72 to 75 %. The diones had a percentage recovery of between 75 and 96 %. In most cases the repeatability of the percentage recovery was better with M5 than B5.

Table 3.9 reports the results from the repeat analysis of the M5 cartridges following washing, with triplicate GC/MS analyses of each eluent, for the prohormones numbered [1] to [6]. Table 3.10 provides the results for the prohormones numbered [7] to [12].

Time pressures and the desire to explore the applicability of the technique with designer steroids meant that repeat analyses, with triplicate GC/MS analyses of each eluent, were not performed for M6, B5 nor B6. The results obtained from the study utilising M5 was deemed as sufficient confirmation of the reproducibility of the assay.

Table 3.9. Repeat analysis of M5 MIPs cartridges, following washing, with triplicate GC/MS analyses of each eluent, for the prohormones numbered [1] to [6].

Number	Prohormone	Injection No.	Recovery M5.1 (%)	Recovery M5.2 (%)	Recovery M5.3 (%)	Average M5 Recovery (%)
1	5(10)- estrene- 3b,17b-diol	1	94.9	122.7	120.8	103.0+/- 15.6
		2	92.9	91.4	124.4	
		3	92.5	84.0	103.6	
		Average	93.5	99.3	116.2	
2	4-estrene- 3b,17b-diol	1	100.0	119.5	114.0	103.1+/-12.7
		2	95.6	95.1	122.7	
		3	84.9	97.9	97.9	
		Average	93.5	104.2	111.5	
3	5(10)- estrene-3,17- dione	1	38.9	103.6	113.4	79.7+/-27.6
		2	55.5	71.2	103.7	
		3	47.4	83.7	100.1	
		Average	47.3	86.2	105.7	
4	4- androstene- 3b,17b-diol	1	108.6	118.1	100.7	104.3+/-7.6
		2	101.9	94.5	112.1	
		3	105.0	95.6	102.4	
		Average	105.1	102.8	105.1	
5	DHEA	1	99.9	108.2	132.6	105.6+/-14.7
		2	102.2	91.9	124.5	
		3	93.6	89.6	108.0	
		Average	98.6	96.6	121.7	
6	5(6)- androstene- 3b,17b-diol	1	72.4	111.7	115.2	87.4+/-18.8
		2	79.8	96.6	97.4	
		3	59.6	75.8	78.0	
		Average	70.6	94.7	96.9	

Table 3.10. Repeat analysis of M5 MIPs cartridges, following washing, with triplicate GC/MS analyses of each eluent, for the prohormones numbered [7] to [12].

Number	Prohormone	Injection No.	Recovery M5.1 (%)	Recovery M5.2 (%)	Recovery M5.3 (%)	Average M5 Recovery (%)
7	5a-androstane-3,17-diol	1	81.6	74.9	82.7	77.5+/-6.2
		2	73.3	69.6	88.6	
		3	81.3	72.9	73.1	
		Average	78.7	72.4	81.5	
8	5a-androstane-3,17-dione	1	103.3	112.8	133.5	106.5+/-15.1
		2	98.5	92.6	124.6	
		3	93.7	89.4	110.4	
		Average	98.5	98.3	122.8	
9	Nandrolone	1	94.7	86.6	116.3	90.8+/-12.7
		2	90.6	74.7	101.6	
		3	90.1	75.9	86.6	
		Average	91.8	79.1	101.5	
10	5(6)-androstene-3,17-dione	1	89.3	99.1	115.6	91.9+/-12.5
		2	89.0	77.2	102.2	
		3	87.0	75.2	92.7	
		Average	88.4	83.8	103.5	
11	Testosterone	1	131.1	134.9	130.9	118.7+/-16.5
		2	117.6	100.3	135.0	
		3	93.3	100.9	125.0	
		Average	114.0	112.0	130.3	
12	1,4-androstadiene-3,17-dione	1	90.0	102.6	115.2	94.2+/-11.6
		2	90.6	79.6	105.2	
		3	90.0	80.4	94.3	
		Average	90.2	87.5	105.0	

It is clear that templating had no significant influence, at the 2 σ level, on the performance of the polymer developed using methacrylic acid (MAA). Apart from 5(10)-estrene-3 β ,17 β -diol, the percentage recovery for templated (M5) was not significantly different to that obtained for the non-templated (B5) polymer. This would suggest that using the methacrylic acid (MAA) as the monomer results in non-specific binding for steroids, presumably through non-covalent electrostatic interactions between the carboxylic acid groups on the polymer and the hydroxyl and ketone groups in the prohormones and matrix species.

Repeating the extractions on all three M5 cartridges, following washing, resulted in an increase in the average percentage recovery of between 10 and 20%, for all prohormone species. GC-MS analyses on each extract were conducted in triplicate. The percentage recovery ranged between 78 and 104 % for the diols; whereas the recovery of DHEA, nandrolone and testosterone, respectively, ranged from 91 to 119 %. The androgenic diones had a percentage recovery of between 92 and 107 %, whilst 5(10)-estrene-3,17-dione was recovered at 25 %. These results would seem to indicate that the washing process was incomplete and would need further investigation if the MIPs were to be re-used; however, as the imprinting conferred no significant improvement over the non-imprinted polymer this was not explored further in this study.

3.2.6. Evaluating the percentage recovery of the prohormones from synthetic urine using cartridges containing imprinted (M6) and non-imprinted (B6) polymers that use 4-VP as the monomer

Having established that imprinting with testosterone had no significant influence on the recovery performance of the polymers, when utilising MAA as the monomer, the

imprinting process was evaluated using 4-vinyl pyridine (4-VP) as the monomer. The percentage recovery of each of the target analytes from synthetic urine was determined using the method specified in Section 2.2.9. Triplicate cartridges, containing either imprinted (M6) or non-imprinted (B6) polymers, were utilised and the results for each prohormone are recorded in Tables 3.11 and 3.12, respectively. Table 3.13 evaluates whether the imprinting had any significant influence on the extraction of the prohormones from urine.

As can be seen in Table 3.11, using the imprinted polymer (M6), the percentage recovery ranged between 70 and 96 % for the diols; whereas the recovery of DHEA, nandrolone and testosterone, respectively, ranged from 68 to 84 %. The diones had a percentage recovery of between 84 and 100%.

Table 3.12 illustrates that the use of the non-imprinted polymer (B6) results in lower percentage recovery of all the prohormones. The percentage recovery ranged between 28 and 37 % for the diols; whereas the recovery of DHEA, nandrolone and testosterone, respectively, ranged from 25 to 37 %. The diones had a percentage recovery of between 37 and 55 %. In most cases, the repeatability of the percentage recovery was comparable for both M6 and B6.

Table 3.11. Percentage recoveries of each of the prohormones based on their extraction from 1 mL of synthetic urine (spiked at 10 ng/mL) on three cartridges of M6.

Number	Prohormone	Recovery M6.1 (%)	Recovery M6.2 (%)	Recovery M6.3 (%)	Average Recovery M6 (%)	Standard Deviation σ_{n-1} (%)
1	5(10)-estrene-3b,17b-diol	80.0	71.0	90.2	80.4	9.7
2	4-estrene-3b,17b-diol	77.6	67.7	63.7	69.7	7.1
3	5(10)-estrene-3,17-dione	125.3	107.1	118.9	117.1	7.9
4	4-androstene-3b,17b-diol	106.7	89.3	90.8	95.6	9.6
5	DHEA	61.2	70.5	73.6	68.4	6.5
6	5(6)-androstene-3b,17b-diol	81.3	78.5	82.2	80.7	1.9
7	5a-androstane-3,17-diol	87.1	70.0	76.1	77.7	8.7
8	5a-androstane-3,17-dione	86.5	92.1	101.7	93.4	7.7
9	Nandrolone	86.7	78.2	83.7	82.9	4.3
10	5(6)-androstene-3,17-dione	94.4	86.4	99.5	93.4	6.6
11	Testosterone	87.3	84.4	79.5	83.7	3.9
12	1,4-androstadiene-3,17-dione	92.6	82.1	79.2	84.6	7.1

Table 3.12. Percentage recoveries of each of the prohormones based on their extraction from 1 mL of synthetic urine (spiked at 10 ng/mL) on three cartridges of the non-templated polymer (B6).

Number	Prohormone	Recovery B6.1 (%)	Recovery B6.2 (%)	Recovery B6.3 (%)	Average Recovery B6 (%)	Standard Deviation σ_{n-1} (%)
1	5(10)-estrene-3b,17b-diol	31.6	37.2	28.3	32.4	4.5
2	4-estrene-3b,17b-diol	33.0	29.1	25.6	29.2	3.7
3	5(10)-estrene-3,17-dione	65.3	46.1	54.5	55.4	9.6
4	4-androstene-3b,17b-diol	30.0	45.6	37.3	37.7	7.8
5	DHEA	24.2	28.6	21.3	24.7	3.6
6	5(6)-androstene-3b,17b-diol	30.7	29.2	23.3	27.7	3.9
7	5a-androstane-3,17-diol	34.4	25.3	23.0	27.5	6.0
8	5a-androstane-3,17-dione	37.6	43.6	30.2	37.1	6.8
9	Nandrolone	38.4	40.2	32.9	37.2	3.8
10	5(6)-androstene-3,17-dione	43.7	41.6	30.0	38.4	7.4
11	Testosterone	34.7	39.7	30.3	34.9	4.7
12	1,4-androstadiene-3,17-dione	38.3	44.9	34.3	39.2	5.3

Table 3.13. Evaluating whether the templating of the 4-vinyl pyridine polymer with testosterone had a significant influence on the extraction of the prohormones.

Number	Prohormone	Average Recovery M6 (%)	Average Recovery B6 (%)	Significant Influence at the 2 σ level?
1	5(10)-estrene-3b,17b-diol	80.4 +/- 19.4	32.4 +/- 9.0	Yes
2	4-estrene-3b,17b-diol	69.7 +/- 14.2	29.2 +/- 7.4	Yes
3	5(10)-estrene-3,17-dione	117.1 +/- 15.8	55.3 +/- 19.2	Yes
4	4-androstene-3b,17b-diol	95.6 +/- 19.2	37.7 +/- 15.6	Yes
5	DHEA	68.4 +/- 13.0	24.7 +/- 7.2	Yes
6	5(6)-androstene-3b,17b-diol	80.7 +/- 3.8	27.7 +/- 7.8	Yes
7	5a-androstane-3,17-diol	77.7 +/- 17.4	27.5 +/- 12.0	Yes
8	5a-androstane-3,17-dione	93.4 +/- 15.4	37.1 +/- 13.6	Yes
9	Nandrolone	82.9 +/- 8.6	37.2 +/- 7.6	Yes
10	5(6)-androstene-3,17-dione	93.4 +/- 13.2	38.4 +/- 14.8	Yes
11	Testosterone	83.7 +/- 7.8	34.9 +/- 9.4	Yes
12	1,4-androstadiene-3,17-dione	84.6 +/- 14.2	39.2 +/- 10.7	Yes

It is clear from the tables that templating the 4-vinyl pyridine monomers with testosterone did have a significant influence on the recovery performance of the polymer. The percentage recovery of all the prohormones using the templated polymer (M6) were significantly higher than those obtained for the non-templated polymer (B6) and comparative to those achieved with MAA. This would suggest that in contrast to the non-specific binding when using methacrylic acid as the monomer, that using 4-vinyl pyridine as the monomer and testosterone as the template results in the formation of specific binding cavities with conformations that are capable of reversibly trapping and releasing the steroids. Presumably, these non-covalent interactions (π - π and dipole-dipole) are between unsaturated bonds on the polymer and those in the ring structure and the substituents of the prohormones.

3.3. Conclusions

In an attempt to overcome the issues associated with traditional analytical techniques, this chapter has explored a novel approach to the extraction, separation, detection and quantification of a cohort of prohormones, spiked into synthetic urine at concentrations that are appropriate for sports testing, as specified by the MRPL values published by WADA.

The method developed extracted unconjugated steroids from the urine using molecular imprinted polymers (MIPs) templated with testosterone. Two variants of the MIPs were developed, using different monomers, and their performances evaluated against non-imprinted versions of the polymers. Uniquely, this work has combined the selectivity of molecular recognition polymers with the high resolving power of two-dimensional gas chromatography/time-of-flight mass spectrometry to enable the identification and quantitation of all the prohormones without the need for derivatisation.

The regulations in place at the time of the study, as prepared by the World Anti-Doping Agency (WADA) (2010 Prohibited List) stated that 46+ compounds (exogenous and endogenous) require close monitoring and were not allowed to be present in concentrations above 10 ng/mL.

The steroid compounds chosen for this study were selected for two reasons. Firstly, they are close structural analogues of testosterone and nandrolone; and are banned substances with respect to WADA's anti-doping watch-list. Some of these compounds act as prohormones and are converted to testosterone and nandrolone in the body. Therefore they are of commercial and legal interest, and as such are a good demonstration of the potential of the developed technique. Secondly the compounds studied share a high level of similarity to testosterone and each other, in terms of charge and functional groups. These similarities allowed a MIP to be developed for a close analogue of structurally similar compounds that is relevant for WADA testing for both exogenous and endogenous steroids.

Based on the findings in this chapter, it is possible to draw the following conclusions:

1. In agreement with previous studies, the identification and quantification of the cohort of twelve prohormones, following derivatisation, is difficult. In addition, the results confirm the following:
 - a. the level of derivatisation is highly variable and many of the derivatised species will co-elute;
 - b. diols and species with a hydroxyl group at the 17- position (*e.g.* nandrolone and testosterone) will, in the main, derivatise;
 - c. diones, will, in the main, not derivatise;

- d. there are exceptions to the above rules and therefore each compound must be evaluated individually;
 - e. underivatised species elute significantly earlier than derivatised species;
 - f. the derivatisation process itself generates species which can interfere with detection of the target species, especially when the sample is analysed using standard single dimension gas chromatography/mass spectrometry.
2. The majority of the underivatised prohormones in a mixed standard solution, were resolved chromatographically using two-dimensional comprehensive chromatography. They also all eluted in a distinct region of the 2D surface plot of the total ion chromatogram (TIC) that was well resolved from the majority of the matrix compounds and column bleed.
3. Whilst 5(10)-estrene-3b,17b-diol and 4-estrene-3b,17b-diol (Peaks #1 & #2) co-eluted in the total ion chromatogram, as did 4-androstene-3b,17b-diol and DHEA (Peaks #4 & #5); all of these species could be resolved and quantified using their diagnostic fragmentation ions.
4. In terms of the elution characteristics of the individual prohormones and the groups of steroids, we could conclude the following:
 - a. 19-norsteroids elute before their androsta(e)ne equivalents (as evidenced by 4-estrene-3b,17b-diol eluting before 4-androstene-3b,17b-diol and nandrolone eluting before testosterone);
 - b. Diols elute before diones (evidenced by the fact that 5 of the first seven species are diols and 3 of the last five species are diones);

- c. The presence of a carbonyl group at the 3- position as well as double bond at the 4-position of Ring A (nandrolone and testosterone) increases retention on the columns, even with a hydroxyl group at the 17-position, this is likely to be due to polarisation or delocalisation of the charge in the C=O and C=C double bonds and their interaction with the delocalised aromatic substituents in the stationary phase;
 - d. Nor-19 steroids, separated only by the location of a double bond (as evidenced by 5(10)-estrene-3b,17b-diol and 4-estrene-3b,17b-diol) will effectively co-elute, but may be distinguished by their diagnostic fragmentation ions;
 - e. Androstenes, separated only by the location of a double bond (as evidenced by 5(6)-androstene-3b,17b-diol and 4-androstene-3b,17b-diol) can be resolved chromatographically;
 - f. In terms of the diones, the elution order is: estrene, androstane, androstene and androstadiene.
5. The integrated area responses measured for a specific quantitation ion for each of the underivatised prohormones in a mixed standard, were linear over the range of 50 to 500 pg injected. This is equivalent to the quantity of material that could be recovered from urine samples spiked at 1 to 10 ng/mL on using the extraction method developed in this thesis. The correlation coefficients were in the range from 0.98 to 0.998, over the concentration range studied.
6. The limits of detection (LOD) and quantitation (LOQ) for each prohormone were estimated by repeat injections of the mixed standard containing 50 pg of each

prohormone injected on column. Based on the results obtained from this standard method ($LOD=3SD$; $LOQ=10\times SD$), it would appear that the twelve prohormones should all be detectable below ~30 pg on column (0.6 ng/mL equivalent in spiked urine); and quantitation should be possible below ~100 pg on column (2.0 ng/mL equivalent in spiked urine). All the prohormones should therefore be detectable below the minimum required performance limit of 10 ng/mL defined by WADA in 2010. There is of course the caveat that most steroids are present in urine in the conjugated form and would probably require hydrolysis prior to MIPs extraction. As previously reported unconjugated steroids make up approximately ~3% of the total steroids present in urine. At the 2010 WADA levels, this would have necessitated a limit of detection of ~15 pg on column. Most of the prohormones in Table 3.5 are estimated to be detectable at around that level.

7. Therefore, with the caveat that a hydrolysis method will probably be required to deconjugate the steroids, prior to MIPs analysis, the method was determined to be fit for its intended purpose.
8. Since this part of the study was completed, WADA lowered its value to 5 ng/mL, for most species, in 2012. Therefore, as it now stands, assuming that the unconjugated steroids are present at 3% of all steroids in a urine sample, then the current method would not be directly applicable to untreated urine samples. This would require a limit of detection of ~ 8 pg on column ($5/33 = 0.15$ ng/mL in urine).
9. It should, however, be noted that the level of detection in this study could be further improved by either: increasing the quantity of urine passed over a larger MIPs

cartridge or by utilising a greater proportion of the sample extracted; for example, by increasing the volume (from 1 μL up to 20 μL) of acetonitrile injected in to the mass spectrometer, through the use of a programmable temperature vapourisation (PTV) injector such as the Optic 4 from ATAS GL, or similar devices from Agilent Technologies or Gerstel. This will require further investigation, as the ability to directly extract a family of unconjugated steroids from urine without treatment, will be a significant step forward. Whether such approaches work will depend on the ability of the MIPs to resolve the target compounds from the matrix peaks that will be present if larger volumes of urine or solvent are used and ultimately the background noise for the quantitation ion in the chromatographic region of interest.

10. The imprinting of a polymer with testosterone, using methacrylic acid (MAA) as the monomer, did not result in a significant increase in the percentage recovery of the prohormones over the non-imprinted polymer; when used to extract the cohort of prohormones from 1 mL of synthetic urine spiked at 10 ng/mL.
11. The imprinting of a polymer with testosterone, using 4-vinyl pyridine (4-VP) as the monomer, resulted in a significant increase in the percentage recovery for all the prohormones when compared with the non-imprinted polymer:
 - a. using the imprinted polymer (M6), the percentage recovery ranged between 70 and 96 % for the diols; whereas, the recovery of DHEA, nandrolone and testosterone, respectively, ranged from 68 to 84 %. The diones had a percentage recovery of between 84 and 100 %.
 - b. using the non-imprinted polymer (B6) the percentage recovery ranged between 28 and 37 % for the diols; whereas the recovery of DHEA,

nandrolone and testosterone, respectively, ranged from 25 to 37 %. The diones had a percentage recovery of between 37 and 55 %, In most cases the repeatability of the percentage recovery was comparable for both M6 and B6.

12. In addition to illustrating that the method is capable of detecting a cohort of unconjugated, underivatised steroids (prohormones) at concentrations that are appropriate for sports testing, it also appears to open up the opportunity for the screening of any banned, forbidden or unknown compounds that is based around the steroid nucleus. The high acquisition rate of the time-of-flight mass spectrometer, coupled with the high signal-to-noise characteristics of the sharp peaks generated in cryogenic comprehensive chromatography, means that it uniquely suitable for the screening of known and unknowns as all the ions between 50-500+ amu can be collected simultaneously. This, in stark contrast to the quadrupole mass spectrometer systems which, due to their scanning nature, must rely on pre-defined windows of selected ions to achieve the desired performance, thus leaving open the opportunity for abuse.

The GC/MS method and the MIPs developed in this study will now be re-applied unchanged, to evaluate whether it can be applied to the detection of designer steroids, developed to deliberately get around the sport testing protocols, in the presence of prohormones.

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- 4. Further validation of the MIPS method coupled to comprehensive gas chromatography / mass spectrometry and its application to designer steroids**

4.1. Introduction

Designer steroids can be defined as a substance or a compound that undergoes a major or minor modification, for the sole purpose of avoiding detection. It is often a synthetic steroid derived by chemical modification from another, usually an anabolic steroid. In contrast to a prohormone, it does not need to be converted to an active form via enzymatic activity within the liver or stomach. Being active from the moment they are orally ingested, they almost immediately contribute to the cocktail of active hormones in the plasma. They also tend to be more potent and have greater anabolic properties than prohormones, and therefore any additional benefits must be considered alongside the more pronounced side effects. They are typically blended with prohormones and other agents, to counteract side effects, in a process known as “stacking”.¹

A minority of athletes are prepared to put their health at risk by using such designer steroids, no matter how small the advantageous anabolic properties may be. This is despite the fact that designer steroids are not usually supported by any published trial data and they have no guarantee as to the quality or long term consequences of taking the product provided by distributors of designer steroids.

In stark contrast, the pharmaceutical industry is regulated by the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK and by the Food and Drug Administration (FDA) in the USA. Therefore, active pharmaceutical ingredients undergo rigorous checks and include specific limitations on impurities and/or degradation products formed during manufacture. For example, the quality guidelines produced by the International Conference for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) require that impurities above 0.05% must be reported, above 0.1% they must be identified, whereas impurities above 0.15% must undergo further toxicology

studies.² These above levels apply for drug substances administered at ≤ 2 g/day, whilst lower percentage values apply for drugs exceeding 2 g/day.² Pharmaceutical products are also assigned a shelf life based upon extensive stability data. No such supporting information is available for designer steroids.

Similarly, the difficulty in gaining ethical approval for *in vivo* metabolism studies of designer steroids has led to a lack of toxicological profiles and efficacy of designer steroids available in peer reviewed journals. What literature that is available, regarding designer steroids, is often out dated, part of a marketed product or available on internet forums where discussions take place between steroid users. Compared to scientific publications, a larger amount of information is freely available on websites, online discussion groups, and forums. The plethora of information includes structure of compounds and discussion on efficacy and safety for potential users. After an exhaustive literature search, some non-peer reviewed articles, that are specific for the designer steroids tested in this chapter, are reported and have been acknowledged in the Reference section accordingly.^{1,4,5,6,8,9,10,11,12,15}

Designer steroids have left unique and defined mark in both the history of Olympic sport and drug testing. Most notably, tetrahydrogestrinone (THG), a previously unknown doping substance, only came to light when a syringe containing the designer steroid was sent to doping officials in 2003.³

THG was developed by Patrick Arnold for the Bay Area Laboratory Co-Operative (BALCO), an American nutritional supplement company, through the hydrogenation of gestrinone over a catalyst.^{3,4} Ultimately, this resulted in the disqualification of several athletes after they were subsequently found to have used the steroid. The two most famous

names embroiled with the BALCO scandal were Barry Bonds and Marion Jones. UK sprinter, Dwain Chambers, was also associated with the scandal.⁵

THG is a highly potent agonist for the androgen and progesterone receptors. It is an order of magnitude more potent than either nandrolone or trenbolone, but has no estrogenic activity.⁶ It has been found to bind to the androgen receptor with similar affinity to dihydrotestosterone.³

In 2004, Catlin *et al.*, were the first to develop and evaluate sensitive and specific methods for the rapid screening of urine samples for underivatized THG, by liquid chromatography/tandem mass spectrometry (LC/MS/MS) and the analysis of the combination trimethylsilyl ether-oxime derivative of THG, by gas chromatography/high-resolution mass spectrometry (GC/HRMS).³

They also confirmed that THG was not detectable in urine by the standard doping control anabolic steroid screening, which uses pertrimethylsilyl derivatives and GC/MS analysis; they went on to state that other screening methods, which rely on alternative derivatisation and GC/MS, are possible but are very cumbersome for THG. In their method, they avoided artefact formation by first incubating the THG, at 65 °C, in a solution of methylhydroxylamine (25 mg/mL) in pyridine. This step was used to prevent the protonation of the oxygen causing enolisation. The trimethylsilyl ether was then prepared by incubating for 1 hour, at 60 °C, in a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide, ammonium iodide and dithioerythritol. A proposed mechanism for the derivatisation process is provided in Figure 4.1.

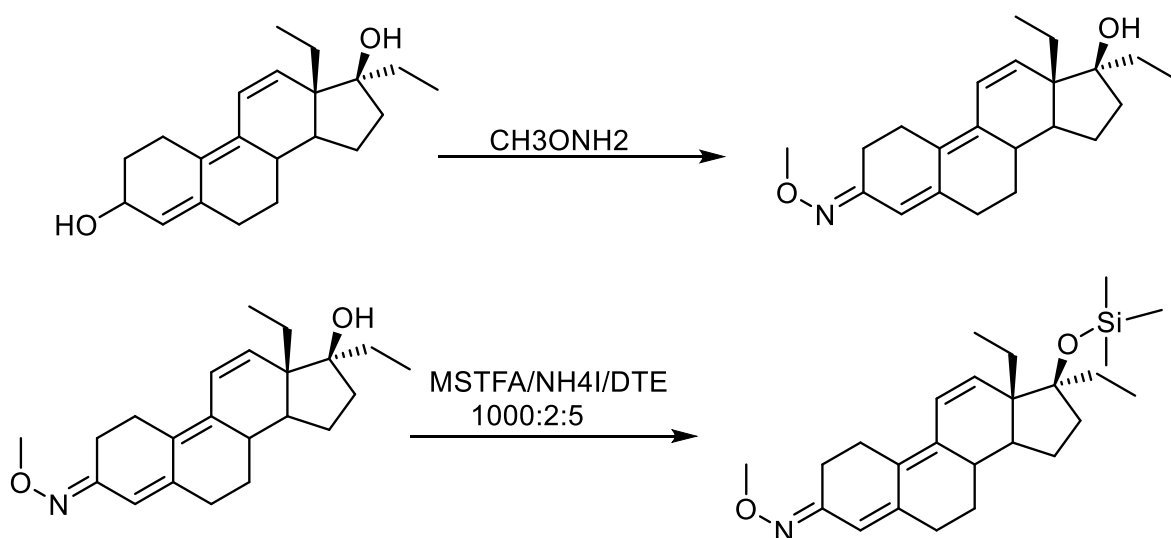


Figure 4.1. A proposed mechanism for the derivatisation of THG.

Whilst they demonstrated that underivatised THG was readily detectable by LC/MS/MS, they recognised that the cost of such a system was a burden on the doping control laboratories and that a simple screening method, by either GC/MS or immunoassay would be more desirable. They also went on to conclude that although GC/MS analysis of THG MOX-TMS by their method is more time consuming, it is a more sensitive and more definitive approach than LC/MS/MS.

The BALCO scandal has shown that the doping screening methods are vulnerable to false negative results, especially when targeted by rogue analysts and athletes. As illustrated in Chapter 3, the range of potential prohormones that could be taken by an athlete is enormous and no single derivatisation method, when coupled with traditional one dimensional gas chromatography/mass spectrometry (GC/MS) analysis, is capable of providing the performance levels required by WADA. This issue is further complicated when one also considers the range of designer steroids that can be manufactured to deliberately avoid derivatisation.

Derivatisation of steroids can be carried out by using silylation or acylation reactions, with the choice of derivatisation mixture dependent on the steroids of interest, as co-elution of the derivatised steroids may occur. As stated above, silylation is the preferred steroid derivatisation approach for GC/MS; with the most popular being N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and the more volatile N-methyl-N-trifluoroacetamide (MSTFA). The use of catalysts is very common in the silylation process and necessary for tertiary hydroxyl groups and enolisation of the carbonyl function. A derivatisation procedure using MSTFA with ethanethiol and ammonium iodide (as an alternative to TMIS) suitable for GC/MS analysis, has been routinely used by the WADA-accredited laboratories for athletic drug testing.⁷ However, this method may lead to the formation of ethyl thio adducts which can cause interpretation problems in the sample analysis. In addition, the failure of some steroids to provide a single reaction product, together with the chemical rearrangement of others, has been reported as a hindrance to official testing laboratories.

As was also shown in Chapter 3, most underivatised prohormones can be extracted from spiked urine samples using molecular imprinted polymers (MIPs) templated with testosterone and then resolved chromatographically by comprehensive gas chromatography / time of flight mass spectrometry (GCxGC/ToFMS). Those prohormones that could not be resolved chromatographically were capable of being resolved using the time of flight mass spectrometer. In contrast to traditional scanning instruments (quadrupole or ion trap), the ability of the ToFMS to collect ions over the full mass range at very high acquisition rates, enables co-eluting peaks to be separated using the deconvolution software and through the extraction of diagnostic fragmentation ions.

Focusing the selectivity of the MIPS on the conformation and electrostatic characteristics of the fused-ring nucleus of testosterone has resulted in a non-targeted extraction process for a family of steroids that share similar characteristics. When coupled with the GCxGC/ToFMS, this enables a full mass spectrum profile to be collected of any steroid present in the urine sample.

This chapter will explore whether the method developed in the previous chapter can be applied, without significant modification, to the detection of designer steroids shown in Figure 4.2. The steroids under investigation are: epitestosterone, Xtren, trenazone, epistane, gestrinone and tetrahydrogestrinone (THG). Methyltestosterone will be used as the internal standard.

Epitestosterone, has been included as it has an identical chemical composition to testosterone, the only difference being the relative orientation of the hydroxyl group at the 17-position. It is anticipated that it will elute from the gas chromatography columns very close to testosterone and therefore should be characterised as part of the method development. Being an inactive epimer of testosterone it only tends to be administered in a cream form, to keep an athlete administering low levels of testosterone below the required T/E ratio used for drug doping violations.

As can be seen from the Figure 4.2, dienedione (Xtren) and dienolone (Trenazone) are both estradienes, differing only by the functional group at the 17-position, with Xtren having a ketone, whereas trenazone has a beta-hydroxyl group. Therefore, Xtren is also a prohormone and a designer steroid, as it must be converted enzymatically to trenazone to become active. Xtren is administered orally whereas trenazone is administered topically, as it has poor oral bioavailability.⁸ The prohormone is reported to be more effective. The

orientation of the double bonds in the fused rings, in contrast to 1,4-androstadiene-3,17-dione and especially testosterone, mean that both Xtren and trenazone will not aromatise and therefore show non-estrogenic properties. In practice, this is thought to result in leaner muscle mass, less water retention and less bloating. Trenazone is reported as having an anabolic/androgenic ratio of 100/10 compared to methyltestosterone. It does, however, show progestenic activity.⁹

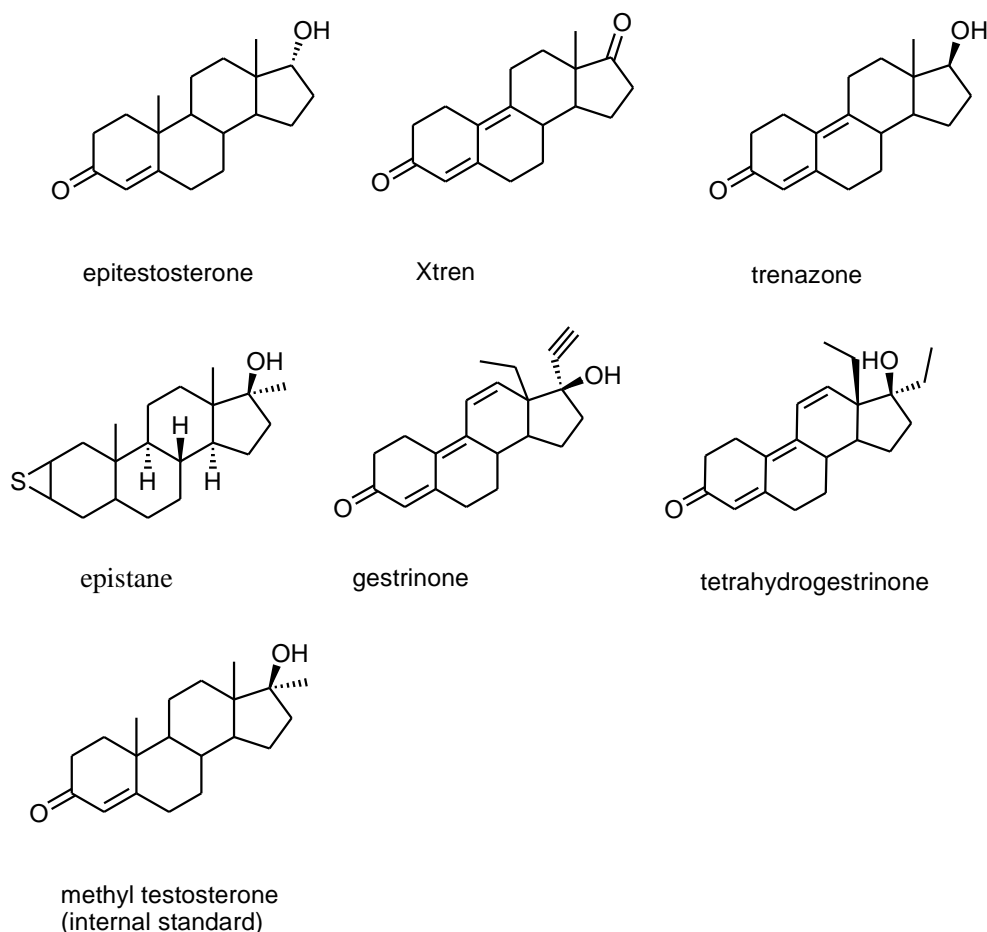


Figure 4.2. The molecular structures of the “designer steroids” selected for evaluating the GCxGC/TOFMS and the molecular imprinted polymer extraction method, developed in Chapters 2 and 3.

Epistane (methylepitiostanol) is the only designer steroid in the cohort that does not have a ketone group at the 3-position; instead, it has a bridging sulphur epimer between the 2- and

3- position, its fused-ring structure is saturated and it has methyl groups attached at the 10-, 13- and 17- positions. The latter introduces steric hindrance in the derivatisation of the beta- hydroxyl group. Whilst epistane does not share the same configuration at the 3- position as the other steroids, it is one of the most popular designer steroids as the addition of the methyl groups make it orally active, preventing digestion, and it is believed to share the non-aromatisation (non-estrogenic) characteristics of epitiostanol.¹⁰ In addition, epistane has an anabolic/androgenic ratio of 660-1000/91-170 relative to methyl testosterone, when taken orally.¹¹

Gestrinone and tetrahydrogestrinone (THG) are both estratrienes, with an ethyl group attached at the 13-position and a beta-hydroxyl group at the 17-position. They only differ by the saturation of the ethynyl (gestrinone) or ethyl (THG) group attached to the 17- position. It is these alkyl functional groups that have been introduced to generate steric hindrance, thus preventing their efficient derivatisation. As with Xtren and trenazone, the locations of the unsaturated bonds in the fused rings of gestrinone and THG mean that they are not aromatising, resulting in their non-estrogenic effect. THG has an anabolic activity ten times greater than that of nandrolone or trenbalone, which will also result in lean muscle mass, and comes with potential side effects such as infertility, acne and hirsutism. It may also suppress the immune system.^{12,13}

Based on the late elution of 1,4-androstadiene-3,17-dione (Boldione) in the prohormone study, it is hypothesised that these multiple saturated species will also be retained significantly by the combination of stationary phases selected.

Methyltestosterone, the 17 alpha-epimer of testosterone, is to be used as an internal standard in this study. The methylation of testosterone at the 17-position has the advantage

of making it orally administrable, as this prevents its digestion. Unfortunately, this small modification has also made it one of the most toxic steroids produced, in particular its toxicity in the liver, both short- and long- term are a major drawback.¹⁴ In addition, it will aromatise to estrogen and be converted to dihydrotestosterone, with all the inherent side-effects associated with both. Other side effects include influencing thyroid hormone production and an increased level of aggression.¹⁵ Of the oral designer steroids, the extreme side effects make it one of the least attractive options.

WADA accredited methods specify the detection of specific steroids at a given minimum required performance levels (MRPL). The MRPL is an analytical parameter of technical performance that the laboratories shall comply with when testing for the presence of a particular prohibited substance, its metabolite(s) or marker(s). In 2010, the MRPL was set at 10 ng/mL for exogenous anabolic androgenous steroids; in 2012 this was reduced to 5 ng/mL.^{16,17} Specific exceptions include dehydrochlormethyltestosterone, methandienone, methyltestosterone and stanozolol, all of which have a MRPL of 2 ng/mL.

4.2. Results and Discussion

4.2.1. Further validation of the GCxGC-MS method

As a consequence of different projects requiring different column configurations, it was necessary to reconfigure the system prior to analysis. In practice, this meant using the same first dimension column, but introducing a new second dimension column. As the shorter, 2 m column, is cut from a longer 10 m column, it is unlikely that the stationary phase coverage will be exactly the same. To ensure that the new configuration was appropriate for the evaluation of the MIPS method, a series of studies were first conducted with steroid

standards to re-characterise the performance of the GCxGC-MS system, both for the new cohort of designer steroids listed earlier and the original cohort of prohormones.

Section 2.2.10, described how the standard mixtures used in this study were prepared. A summary of the composition of each of the solutions is provided in Table 4.1.

Table 4.1. Summary of the concentrations of the steroids present in the mixed designer and prohormone standards used during the characterization studies

Standard name	Designer Steroid Concentration (ng/mL)	Prohormone Concentration (ng/mL)	Internal Standard Concentration (ng/mL)
DS#1	500	-	-
DSP#1	250	250	-
DSPI#1	100	100	167
DSPI#2	50	50	167
DSPI#3	25	25	167

Initially, the elution characteristics of the designer steroids were evaluated in isolation from the prohormones and the internal standard, in order to: a) identify if they could be detected, b) determine the region in which they elute, and c) to evaluate any potential co-elutions with the known prohormone elution region.

As can be seen in Figure 4.2, epitestosterone [13] elutes close to the testosterone retention time. This is not unexpected given that it is the epimer of testosterone, with only the

orientation of the hydroxyl group at the 17- position being different. Xtren [14] and trenazone [15] are both estradienes, and in both dimensions they elute later than the prohormones characterised in the previous chapters. Epistane [16], which has the bridging thiol group instead of a ketone or hydroxyl group at the 3-position, is retained even longer on both columns.

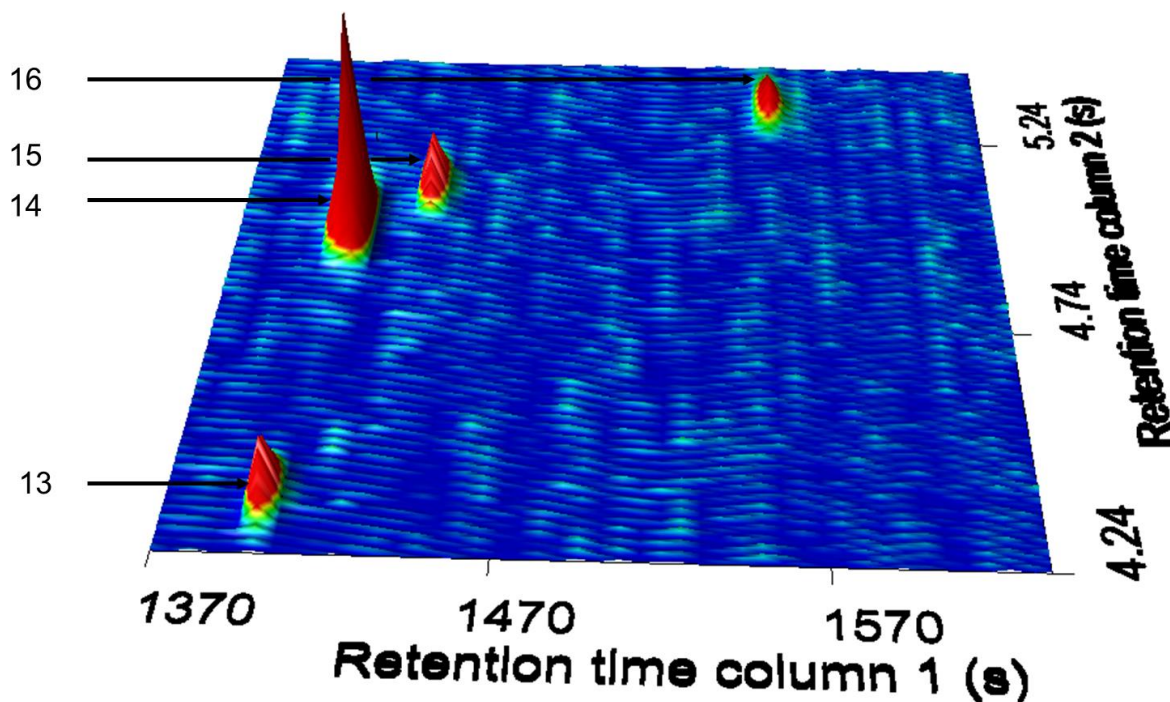


Figure 4.3. A 2D surface plot [TIC] illustrating the elution regions for the underivatised designer steroids (epitestosterone [13], Xtren [14], trenazone [15] and epistane [16]).

Gestrinone [17] and THG [18], cannot be seen in Figure 4.3, this is because they are both retained even further and do not elute within the modulation time. This is not an issue, as can be seen in Figure 4.4, they elute off the second column in a very clean part of the chromatogram, prior to where the non-polar column bleed and matrix species elute. This effect is known as “wrap around” and can either be allowed to continue or can be removed through an increase in the modulation time. Whilst increasing the modulation time, from

the current six seconds, (maximum of ~10 s) would bring gestrinone and THG within this window (they would then be found to the top right of the traces) it would also increase the size of the slices taken of the first dimension eluent which could result in a reduction in the resolution of other compounds.

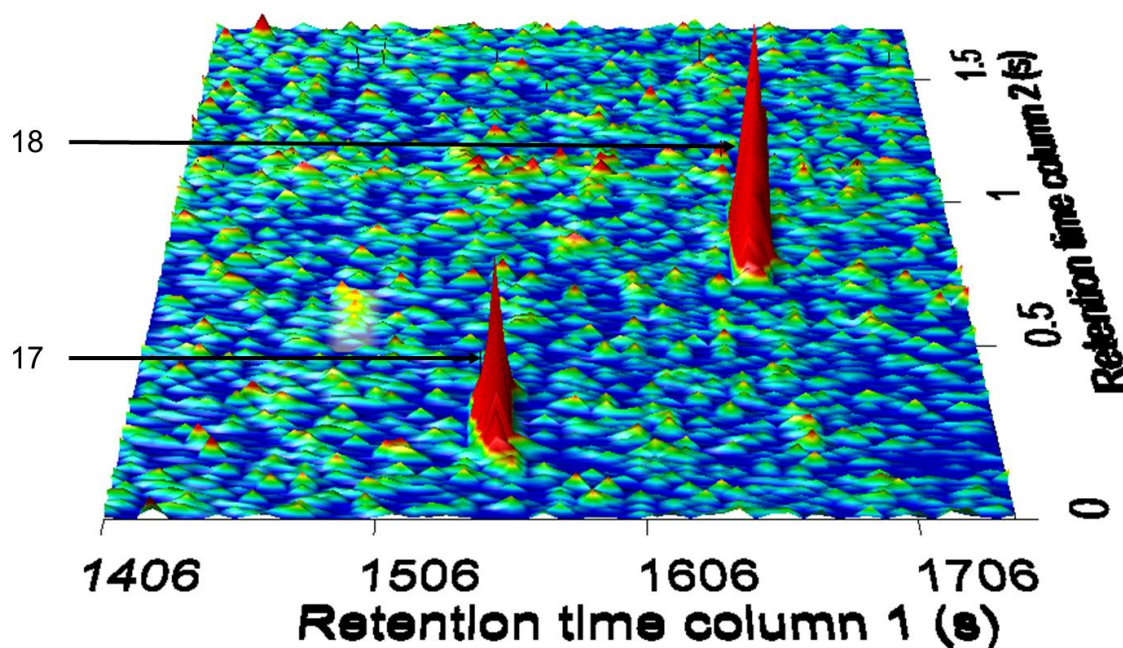


Figure 4.4. A 2D surface plot [TIC] illustrating the elution regions for the underivatised designer steroids (gestrinone [17] and THG [18]). Note the retention times in the second dimension, which indicate they have eluted after the modulation period, a process known as “wrap around”.

The traces in Figures 4.3 and 4.4 were obtained from a 1 μ L splitless injection of a mixed designer steroid standard solution containing 500 ng/mL of each in acetonitrile; this is equivalent to 500 pg (0.5 ng) of each steroid being injected splitless on to the column. Based on the existing extraction method and assuming complete recovery, this would be consistent with having an initial concentration level of 10 ng/mL of each steroid present in 1 mL of urine, the equivalent of the detection levels required by the 2010 WADA Prohibitive List.

A better illustration of the elution regions for the designer steroids, relative to the prohormones, is provided in Figure 4.5. This surface plot is taken from a 1 μ L injection of the mixed prohormone and designer steroid standard (DSP#1) at a concentration of 250 ng/mL of each. 250 pg on column being equivalent to 5 ng/mL in urine, the detection levels specified in the 2012 WADA Prohibitive List.

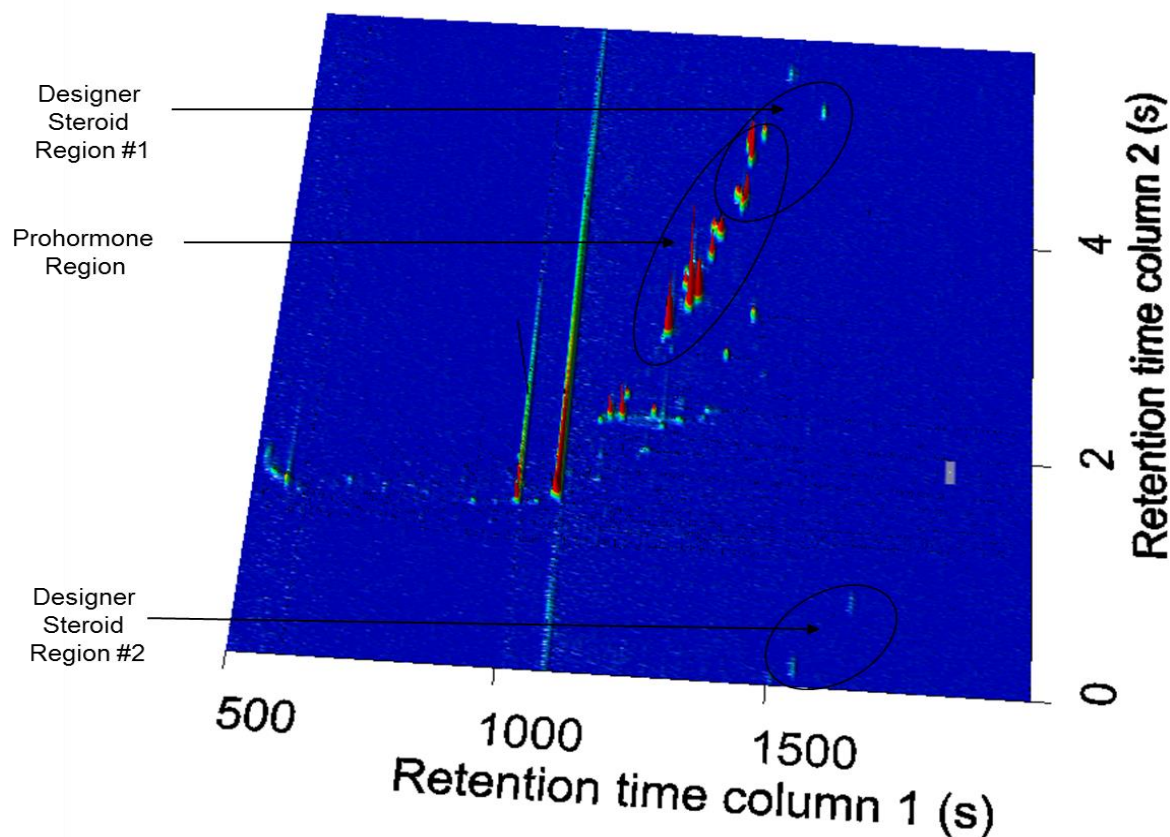


Figure 4.5. A 2D surface plot [m/z 99] illustrating the elution regions for:

- i) Prohormones
- ii) Designer Steroids #1 (epitestosterone, Xtren, trenazone, epistane)
- iii) Designer Steroids #2 (gestrinone, THG)

Figure 4.5 shows that epitestosterone, Xtren, trenazone, and epistane elute in a region that partially overlaps with the prohormone region. As predicted, epitestosterone elutes very close to testosterone. They pretty much co-elute, as will be illustrated in the following figures. Xtren and trenazone both have two double bonds in the fused ring structure and

therefore their interaction with the aromatic groups in the stationary phase of the gas chromatography columns is stronger than that of the prohormones that have greater saturation. Similarly, gestrinone and THG have three double bonds, one in each of the rings A, B and C and their interaction with the aromatic groups of the stationary phase are the greatest, and actually results in them being retained beyond the modulation period of six seconds. They uniquely elute in a region to the bottom right hand side of the trace.

Figures 4.6 and 4.7 identify the individual prohormones and the four steroids in the first designer steroid region. In Figure 4.6, the upper, contour plot allows the relative locations of each of the compounds in each dimension to be identified, *i.e.* their elution times on each column. The lower, surface plot gives an indication of the relative peak abundances of each of the compounds. Figure 4.7, provides a series of surface plots with a different perspective of the closely eluting peaks.

All the peaks result from the same 1 μ L injection of a mixed standard solution, with each steroid present at a concentration of 250 ng/mL (as before, 250 pg being the equivalent to the detection levels required by the 2012 WADA Prohibitive List). Once again, as previously explained in the Chapter 3, the caveat is that these steroid standard are present in the unconjugated form. The fragment ion at m/z 99 was found to be the best ion to illustrate the presence of all the steroids.

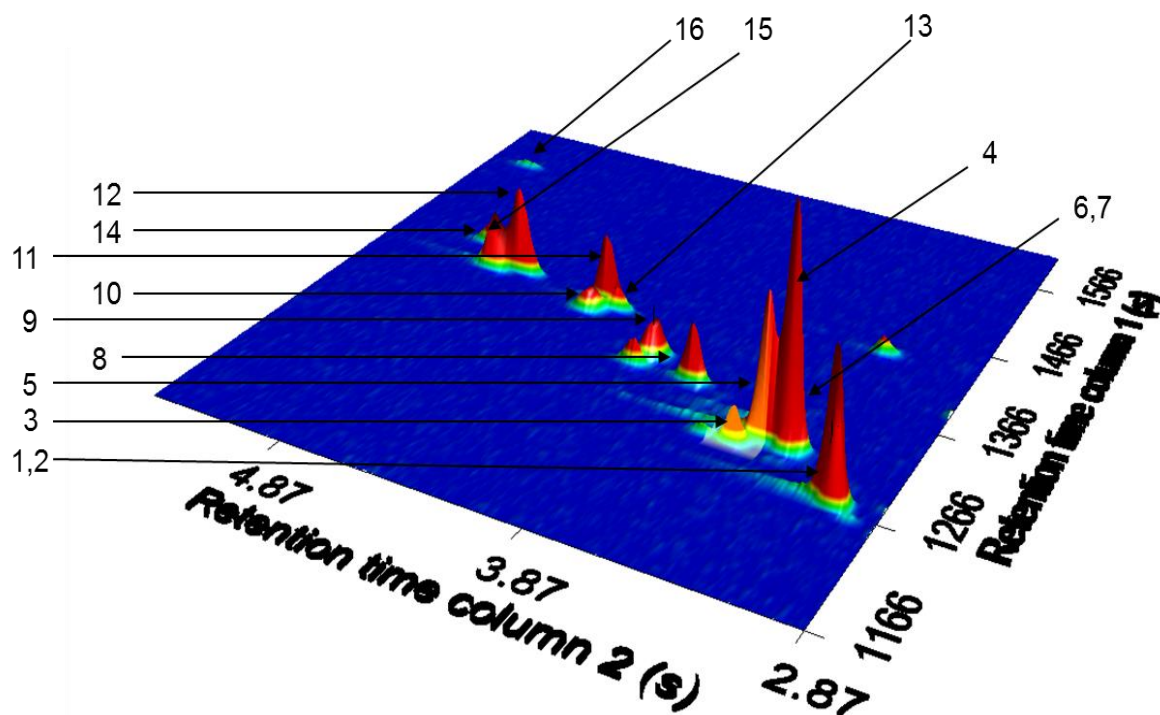
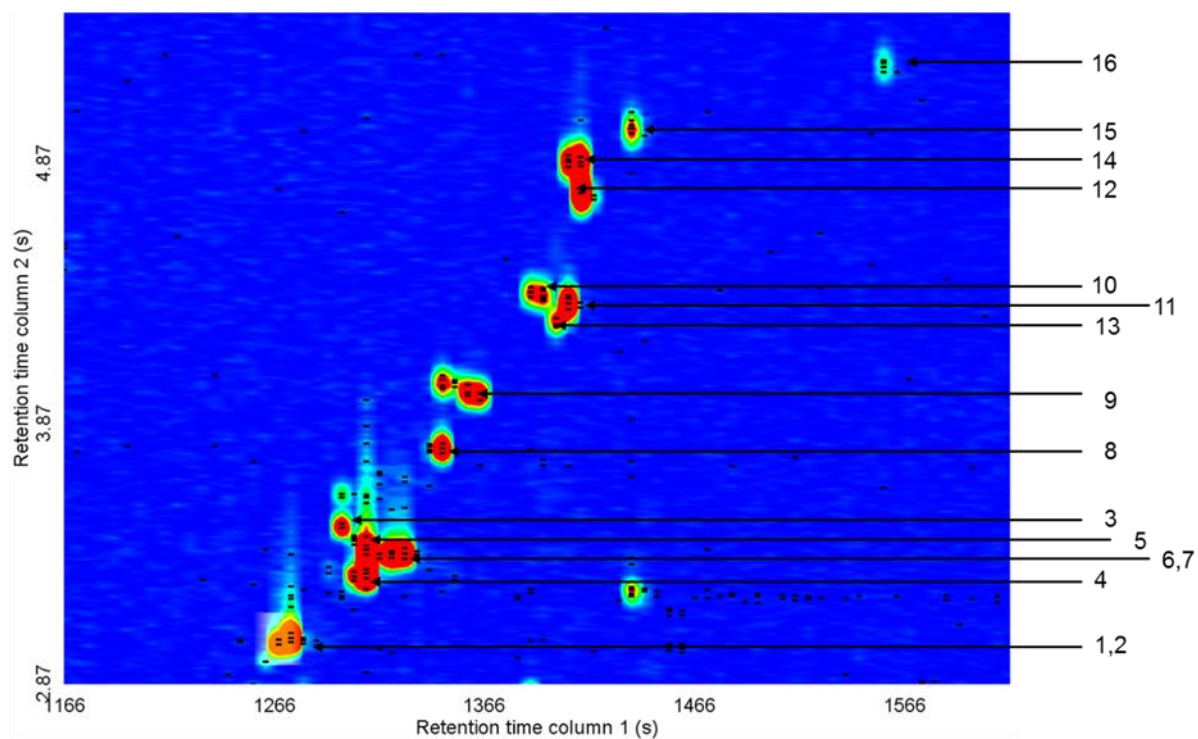


Figure 4.6. A 2D contour plot and surface plot [$m/z = 99$] identifying the prohormones and the designer steroids present in a 1 μL injection of a mixed solution of steroids, each at a concentration of 250 ng/mL.

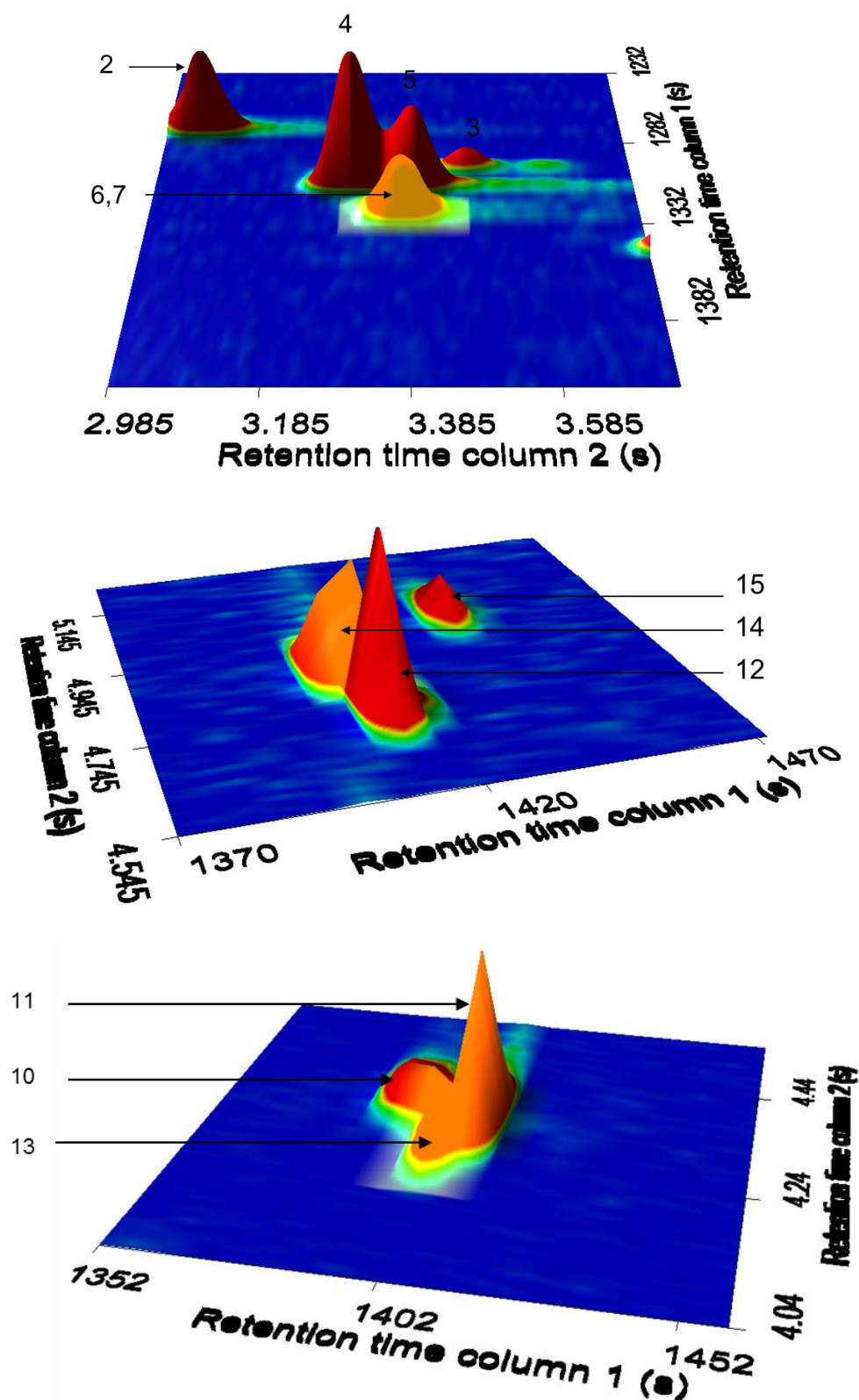


Figure 4.7. Alternative perspectives of the previous 2D surface plot [$m/z = 99$] clarifying the identities and locations of the prohormones and the designer steroids present in a 1 μL injection of a mixed solution containing each of the steroids at a concentration of 250 ng/mL (5 ng/mL).

Similar plots can be drawn for each of the steroids in each of the mixed standard solutions at each concentration. From these chromatograms, quantitative measurements such as the retention times of the peak apex, the peak areas and signal to noise can be determined to evaluate the analytical technique. As was shown in the previous chapter, assessing the chromatographic resolution and reproducibility, the limits of detection and quantitation and linearity of the responses for the individual steroids is best performed using diagnostic fragmentation ions. Ideally, these ions are selected as being unique or significantly more abundant in the steroid of interest and are selected from the full fragmentation pattern available for each data point. Proposed mechanisms for the formation of these fragments are provided in Appendix A1.2 and A1.3. For example, gestrinone's fragmentation is most easily recognized by the loss of the D ring, resulting in a highly diagnostic ion forming at m/z 227. The removal of water (18 Da) from the molecular ion also results in the fragment at m/z 291. Homolytic cleavage at position 13, results in the formation of a fragment at m/z 261.

A summary of the first dimension retention times for each of the steroids, at each concentration, is provided in Table 4.2. It should be noted that the modulation of the eluent from the first gas chromatography column onto the second column takes place every six seconds, effectively dividing the chromatographic trace in to a series of six second slices. It is possible for the modulation to take place part of the way through an eluting peak, which results in the peak being divided over two slices. For species that were found in more than one modulation, the retention time was assigned to the slice with the greatest signal to noise ratio. The criteria for the assignment of a peak was that the signal to noise ratio for a particular steroid had to be above 3. In addition to the retentions times in the first

dimension, the table includes the fragmentation ion selected for each steroid. As a result of the mixed standards prepared, the designer steroids were analysed a total of eleven times, the prohormones were analysed a total of ten times, whereas the internal standard was analysed nine times. Triplicate analyses were performed at 100, 50 and 25 pg, respectively. Based on the standard extraction methodology, these on column injections, assuming 100% recovery, would be the equivalent to the material recovered from urine samples with a concentration range of between 2.0 and 0.5 ng/mL, well below the detection levels required by WADA for steroids. The exception is the internal standard, methyl testosterone, which has a MRPL of 2 ng/mL.

It is worth noting that the identification of a suspected substance by WADA is usually undertaken by matching chromatographic retention time between an unknown and a certified reference standard. Gas chromatography is coupled with MS to provide additional certainty for the positive identification of a suspected finding, over cheaper, non-specific detectors, such as flame ionisation detectors (FID). However, given the detection levels required and the complexity of the sample matrix, scanning MS systems, such as quadrupole GC-MS systems, use selected ion monitoring (SIM) windows to detect and quantify species. With a limited number of ions available in each window, characterising retention time is vital in the evidence against a doping suspect. Automated chromatographic data systems use retention time windows to integrate and identifying suspected samples within an automated platform. The coupling of comprehensive chromatography with a time-of-flight MS, reduces the reliance on retention time matching as compounds can be separated chromatographically, reducing the chance of co-elution, before being identified using a full fragmentation patterns available from the high acquisition TOFMS.

Table 4.2. A summary of the first dimension retention times (s) of the prohormones, designer steroids and the internal standard determined throughout the linearity studies with steroid standards.

Steroid No.	Steroid Name	Ion m/z (Th)	500 pg	250 pg	100 pg (1)	100 pg (2)	100 pg (3)	50 pg (1)	50 pg (2)	50 pg (3)	25 pg (1)	25 pg (2)	25 pg (3)
1	5(10)-estrene-3b,17b-diol	225	-	1268	1274	1274	1268	1268	1268	1268	1268	1268	1268
2	4-estrene-3b,17b-diol	218	-	1274	1274	1274	1274	1274	1274	1274	1274	1274	1274
3	5(10)-estrene-3,17-dione	215	-	1298	1298	1298	1298	1298	1298	1298	1298	1298	1298
4	4-androstene-3b,17b-diol	220	-	1310	1310	1310	1310	1310	1310	1310	1310	1310	1310
5	DHEA	255	-	1310	1310	1310	1310	1310	1310	1310	1310	1310	1310
6	5(6)-androstene-3b,17b-diol	205	-	1322	1322	1322	1322	1322	1322	1322	1322	1322	1322
7	5a-androstane-3b,17b-diol	292	-	1328	1328	1328	1328	1328	1328	1328	1328	1328	1328
8	5a-androstane-3,17-dione	217	-	1346	1346	1346	1346	1346	1346	1346	1346	1346	1346
9	Nandrolone	110	-	1358	1364	1352	1358	1358	1358	1358	1358	1358	1358
10	5(6)-androstene-3,17-dione	124	-	1388	1394	1394	1388	1388	1388	1388	1388	1388	1388
11	Testosterone	124	-	1406	1412	1406	1406	1406	1406	1406	1406	1406	1406
12	1,4-androstadiene-3,17-dione	122	-	1412	1418	1412	1412	1412	1412	1412	1412	1412	1412
13	Epitestosterone	147	1400	1400	1400	1400	1400	1400	1400	1400	1400	1400	1400
14	Xtren	174	1412	1412	1412	1412	1406	1406	1406	1406	1406	1406	1406
15	Trenazone	135	1436	1436	1442	1436	1436	1436	1436	1436	1436	1436	1436
16	Epistane	255	1556	1556	1556	1556	1556	1556	-	-	-	-	-
17	Gestrinone	129	1550	1550	1550	1550	1550	1550	-	1550	-	-	-
18	THG	227	1652	1652	1664	1658	1652	1652	1652	1652	1652	-	-
19	Methyltestosterone	91	-	-	1430	1430	1424	1424	1424	1424	1424	1424	1424

For species that were found in more than one modulation in the first dimension the peak was assigned to the slice that had the highest apex present. The criterion for the assignment of a peak was that the signal to noise for any quantitation ion had to be above 3.

It is clear from Table 4.2 that the method is highly reproducible with the apex of the peak being assigned either to the same or consecutive modulations in the majority of cases. Only nandrolone and THG are found to be assigned in three different modulations. Eight analyses of nandrolone give the same retention time; the two outliers are found at one modulation either side. In the case of THG, seven analyses are identical with one outlier being one modulation away from the mean, and the second being two modulations. The WADA criteria for traditional GC-MS methods using selected ion monitoring, require that the retention time of the analyte shall not differ by more than 1% or 0.2 minutes (whichever is smaller) from that of the same substance in a spiked urine sample or reference material analysed contemporaneously. The assignment of the majority of the steroid species in to one of two slices is more than consistent with the WADA requirement and even nandrolone and THG are within the required 12 s window.

It is also clear from the same table that peaks are identified (above the signal to noise threshold) for all the prohormones and they are assigned a retention time at all concentrations. This is also the case for epitestosterone, Xtren and trenazone, but not for epistane, gestrinone and THG. This issue will be returned to during the discussion on the signal to noise ratios obtained for all species. Potential mechanisms for the formation of the fragmentation ions for each of the prohormones and designer steroids are provided in Appendix A1.2 and A1.3, respectively.

Table 4.3 summarises all the retention times determined for each of the steroids in the second dimension over the range of mixed standard samples analysed, whilst Table 4.4 summarises the relative standard deviation (RSD(%)) for the second dimension retention times for each steroid, at each concentration.

Table 4.3. A summary of the retention times(s) obtained for the prohormones, designer steroids and the internal standard in the second dimension, as determined throughout the linearity studies with steroid standards.

Steroid No.	Steroid Name	Ion m/z (Th)	500 pg	250 pg	100 pg (1)	100 pg (2)	100 pg (3)	50 pg (1)	50 pg (2)	50 pg (3)	25 pg (1)	25 pg (2)	25 pg (3)
1	5(10)-estrene-3b,17b-diol	225	-	3.045	3.300	3.2600	3.020	3.010	3.010	3.010	3.015	2.995	3.010
2	4-estrene-3b,17b-diol	218	-	3.050	3.265	3.2400	3.020	3.025	3.015	3.025	3.025	3.030	3.030
3	5(10)-estrene-3,17-dione	215	-	3.475	3.765	3.7100	3.450	3.435	3.455	3.455	3.460	3.440	3.470
4	4-androstene-3b,17b-diol	220	-	3.300	3.555	3.4950	3.255	3.250	3.270	3.265	3.260	3.250	3.250
5	DHEA	255	-	3.390	3.670	3.6050	3.350	3.345	3.365	3.360	3.365	3.350	3.355
6	5(6)-androstene-3b,17b-diol	205	-	3.375	3.650	3.5800	3.335	3.345	3.340	3.345	3.365	3.335	3.340
7	5a-androstane-3b,17b-diol	292	-	3.375	3.640	3.5650	3.340	3.350	3.350	3.355	3.350	3.355	3.345
8	5a-androstane-3,17-dione	217	-	3.780	4.075	4.0000	3.750	3.755	3.765	3.760	3.750	3.750	3.745
9	Nandrolone	110	-	3.995	4.295	4.2600	3.945	3.970	3.960	3.975	3.985	3.985	3.950
10	5(6)-androstene-3,17-dione	124	-	4.380	4.705	4.5900	4.360	4.365	4.375	4.360	4.345	4.340	4.345
11	Testosterone	124	-	4.335	4.655	4.5650	4.310	4.305	4.315	4.320	4.325	4.325	4.300
12	1,4-androstadiene-3,17-dione	122	-	4.760	5.095	5.0050	4.295	4.300	4.725	4.750	4.745	4.730	4.715
13	Epitestosterone	147	4.370	4.255	4.580	4.500	4.220	4.235	4.235	4.255	4.260	4.245	4.235
14	Xtren	174	4.980	4.880	5.245	5.145	4.855	4.850	4.870	4.870	4.870	4.860	4.860
15	Trenazone	135	5.110	5.010	5.350	5.255	4.955	4.960	4.970	4.970	4.995	4.980	4.965
16	Epistane	255	5.350	5.245	5.610	5.485	5.205	5.250	-	-	-	-	-
17	Gestrinone	129	0.265	0.120	0.415	0.480	0.160	0.206	-	0.251	-	-	-
18	THG	227	0.840	0.735	1.085	0.997	0.729	0.705	0.730	0.725	1.770	-	-
19	Methyltestosterone	91	-	-	4.630	4.550	4.295	4.300	4.310	4.320	4.310	4.315	4.300

For species that were found with more than one peak marker in the second dimension the peak was assigned to the marker that had the greater signal present. The criterion for the assignment of a peak was that the signal to noise for any quantitation ion had to be above 3.

Table 4.4. A summary of the relative standard deviation (RSD(%)) for the retention times obtained for the second dimension, as determined throughout the linearity studies with steroid standards.

Steroid No.	Steroid Name	RSD 2nd Dimension Retention Time [100 pg] (%)	RSD 2nd Dimension Retention Time [50 pg] (%)	RSD 2nd Dimension Retention Time [25 pg] (%)
1	5(10)-estrene-3b,17b-diol	4.7	0.0	0.3
2	4-estrene-3b,17b-diol	4.2	0.2	0.1
3	5(10)-estrene-3,17-dione	4.6	0.3	0.4
4	4-androstene-3b,17b-diol	4.6	0.3	0.2
5	DHEA	4.8	0.3	0.2
6	5(6)-androstene-3b,17b-diol	4.7	0.1	0.5
7	5a-androstane-3b,17b-diol	4.4	0.1	0.1
8	5a-androstane-3,17-dione	4.3	0.1	0.1
9	Nandrolone	4.6	0.2	0.5
10	5(6)-androstene-3,17-dione	3.9	0.2	0.1
11	Testosterone	4.0	0.2	0.3
12	1,4-androstadiene-3,17-dione	9.1	5.5	0.3
13	Epitestosterone	4.3	0.3	0.3
14	Xtren	4.0	0.2	0.1
15	Trenazone	4.0	0.1	0.3
16	Epistane	3.8	-	-
17	Gestrinone	48.1	-	-
18	THG	19.8	1.8	-
19	Methyltestosterone	3.9	0.2	0.2

As can be seen from the preceding tables, the majority of the steroids show good reproducibility for the retention times in the second dimension, with the percentage relative standard deviation being generally less than 5 %. The greatest variation in the assignment of the peak marker in the second dimension occurs for all steroids during injections of 100 pg. This may be related to the fact that it is the highest concentration under study and the variability is significantly greater than that seen at 50 or 25 pg. The reproducibility of the assignment of the apex of the peak might be improved by either: a) reducing the data acquisition rate from its current 200 Hz, or b) applying further data smoothing, post collection. Reducing the acquisition rate would also improve the signal to noise and may also improve the limit of detection.

The two species that show the greatest variability in the second dimension retention times are gestrinone and THG. Since these are the two species that elute outside of the six second modulation window it isn't suprising that they show the greatest variability. Gestrinone in particular is influenced by the fact that it is eluting from the second column, just as the modulation period terminates. Lengthening the modulation period would prevent the existing issues with the variability in elution times in the second dimension and would result in gestrinone and THG being located close to epistane, to the top right hand side of the 2D surface plot. An evaluation would need to be conducted in terms of the influence on the detection limits of other steroids, should the modulation period be increased to the ~9 seconds necessary to elute both gestrinone and THG.

The assignment of the retention times of each of the steroid compounds, at different concentrations, is dependent on the signal to noise ratio for each compound being above a minimum value of 3. Table 4.5 summarises the signal to noise determined for the quantitation ion for each steroid in each sample.

Table 4.5. A summary of the signal to noise values obtained for the prohormones, designer steroids and the internal standard, as determined throughout the linearity studies with steroid standards

No.	Steroid Name	Ion m/z (Th)	500 pg	250 pg	100pg (1)	100pg (2)	100pg (3)	50 pg (1)	50 pg (2)	50 pg (3)	25 pg (1)	25 pg (2)	25 pg (3)
1	5(10)-estrene-3b,17b-diol	225	-	53.0	29.4	27.5	21.2	9.3	7.0	7.9	5.9	4.3	4.5
2	4-estrene-3b,17b-diol	218	-	153.1	40.2	37.9	39.7	14.3	17.1	10.5	9.2	10.1	8.6
3	5(10)-estrene-3,17-dione	215	-	109.3	43.1	46.7	42.7	10.5	8.6	9.1	4.3	5.7	3.9
4	4-androstene-3b,17b-diol	220	-	476.0	139.4	136.3	112.0	30.1	30.2	24.8	20.7	22.1	14.6
5	DHEA	255	-	225.8	46.0	47.6	47.8	26.7	17.8	17.3	13.5	11.1	11.5
6	5(6)-androstene-3b,17b-diol	205	-	126.3	48.1	35.9	34.4	11.6	10.9	10.3	6.8	4.3	5.0
7	5a-androstane-3b,17b-diol	292	-	169.9	30.7	38.5	46.6	18.4	12.9	17.4	12.4	10.4	10.3
8	5a-androstane-3,17-dione	217	-	217.9	51.2	55.0	49.3	21.6	19.6	16.9	12.6	11.7	11.7
9	Nandrolone	110	-	307.6	199.5	198.8	142.3	65.1	63.8	53.5	33.2	34.6	31.3
10	5(6)-androstene-3,17-dione	124	-	158.5	119.9	88.9	94.7	32.0	27.1	33.7	24.7	23.3	16.5
11	Testosterone	124	-	964.8	276.7	259.4	272.2	131.3	100.5	99.0	81.6	80.5	73.3
12	1,4-androstadiene-3,17-dione	122	-	2371.3	376.0	739.9	764.9	352.3	306.5	265.7	196.0	189.9	186.1
13	Epitestosterone	147	85.3	73.1	16.4	18.8	24.8	10.2	8.9	9.7	7.3	4.5	6.8
14	Xtren	174	1057	660.5	293.1	290.9	323.0	79.2	72.9	59.3	44.4	36.9	31.0
15	Trenazone	135	136.2	108.8	39.3	27.5	42.8	14.1	13.6	10.5	7.9	7.5	8.3
16	Epistane	255	42.8	21.9	7.2	6.4	9.5	3.0	-	-	-	-	-
17	Gestrinone	129	30.5	18.3	8.4	18.1	12.9	3.4	-	3.9	-	-	-
18	THG	227	43.5	30.2	6.6	7.9	11.8	7.1	4.6	4.7	3.4	-	-
19	Methyltestosterone	91	-	-	51.4	46.4	35.5	33.0	26.9	24.8	35.1	35.6	33.7

Table 4.5 lists all the values for the signal to noise determined for each steroid, at each of the masses injected, along with the fragmentation ion used. The signal to noise values are then used to determine the limit of detection and quantitation for each steroid. Steroids that have a signal to noise greater than 3, are assigned as being detectable, those with a signal to noise of greater than 5 are considered quantifiable.

It is interesting that the limits of detection predicted for the prohormones, based on the replicate analyses at 50 pg reported in Chapter 3, are pretty much confirmed by the findings listed in the table at 25 pg. All the prohormones are detectable, above the signal noise limit, at 25 pg, which would be equivalent to a concentration in urine of 0.5 ng/mL.

The designer steroids, epitestosterone, Xtren and trenazone are all detectable at 25 pg. Xtren, in particular, has a very strong signature at m/z 174. THG is detectable in all three injections at 50 pg and just detectable in one injection at 25 pg. Gestrinone is not detectable at 25 pg but is just detectable in two of the three injections at 50 pg. Epistane is not detectable at 25 pg and is only found in one of the injections at 50 pg.

Figure 4.8, provides the confirmatory 2D surface plots for epitestosterone (m/z 147), when injected at 100, 50 and 25 pg. Having the same chemical composition as testosterone and only deviating in the orientation of the hydroxyl group at the 17- position, it is not surprising that it partially co-elutes with testosterone, as previously shown in Figure 4.7.

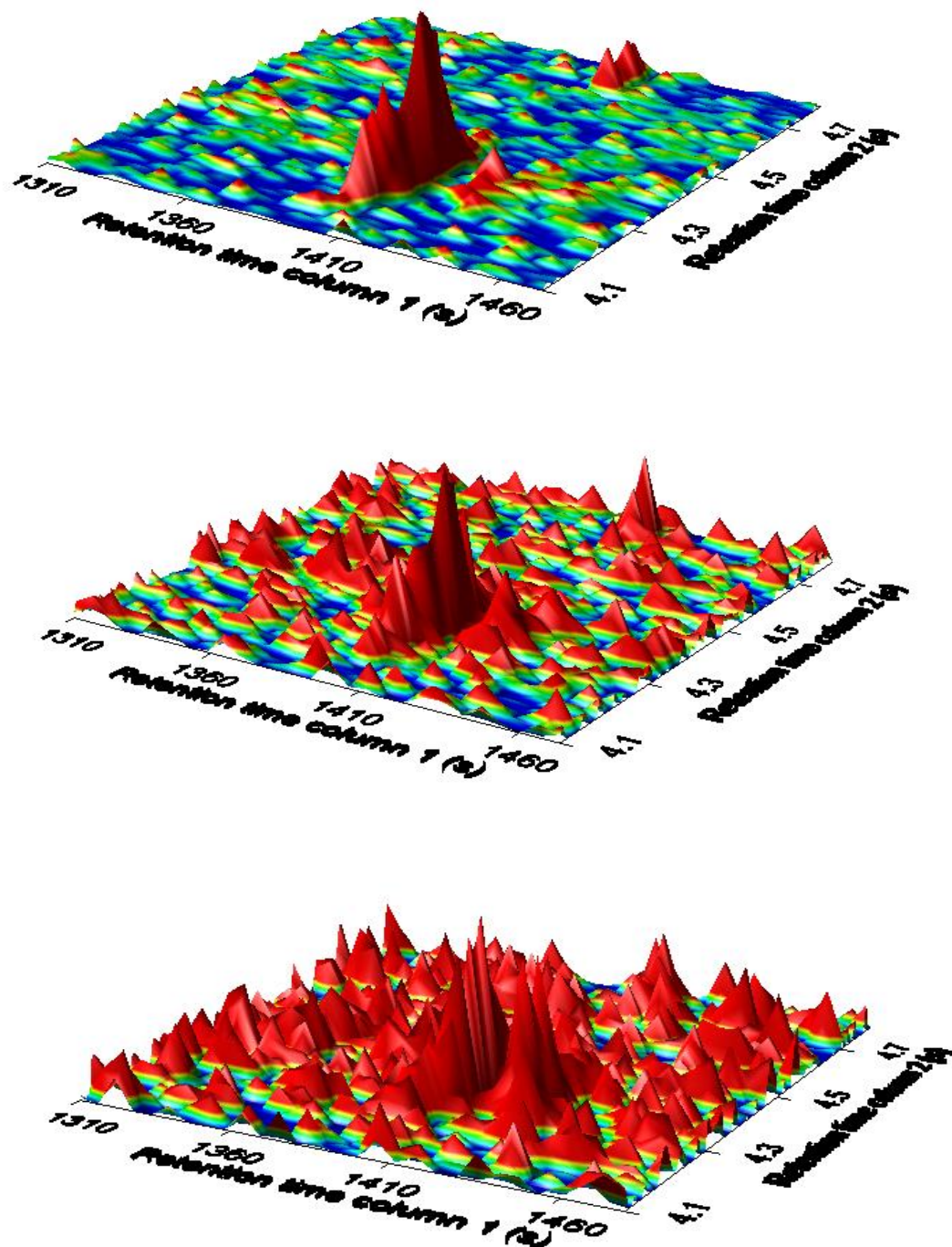


Figure 4.8. 2D surface plots illustrating the responses obtained for epitestosterone using its diagnostic fragmentation ion ($m/z = 147$) at 100, 50 and 25 pg, respectively.

Whilst epitestosterone was predictably difficult to resolve from testosterone, other designer steroids were shown to be easier to identify. In particular, as can be seen in Figures 4.9 - 4.10, the estradienes: dienedione (Xtren) and dienolone (trenalone) are well resolved from the prohormones.

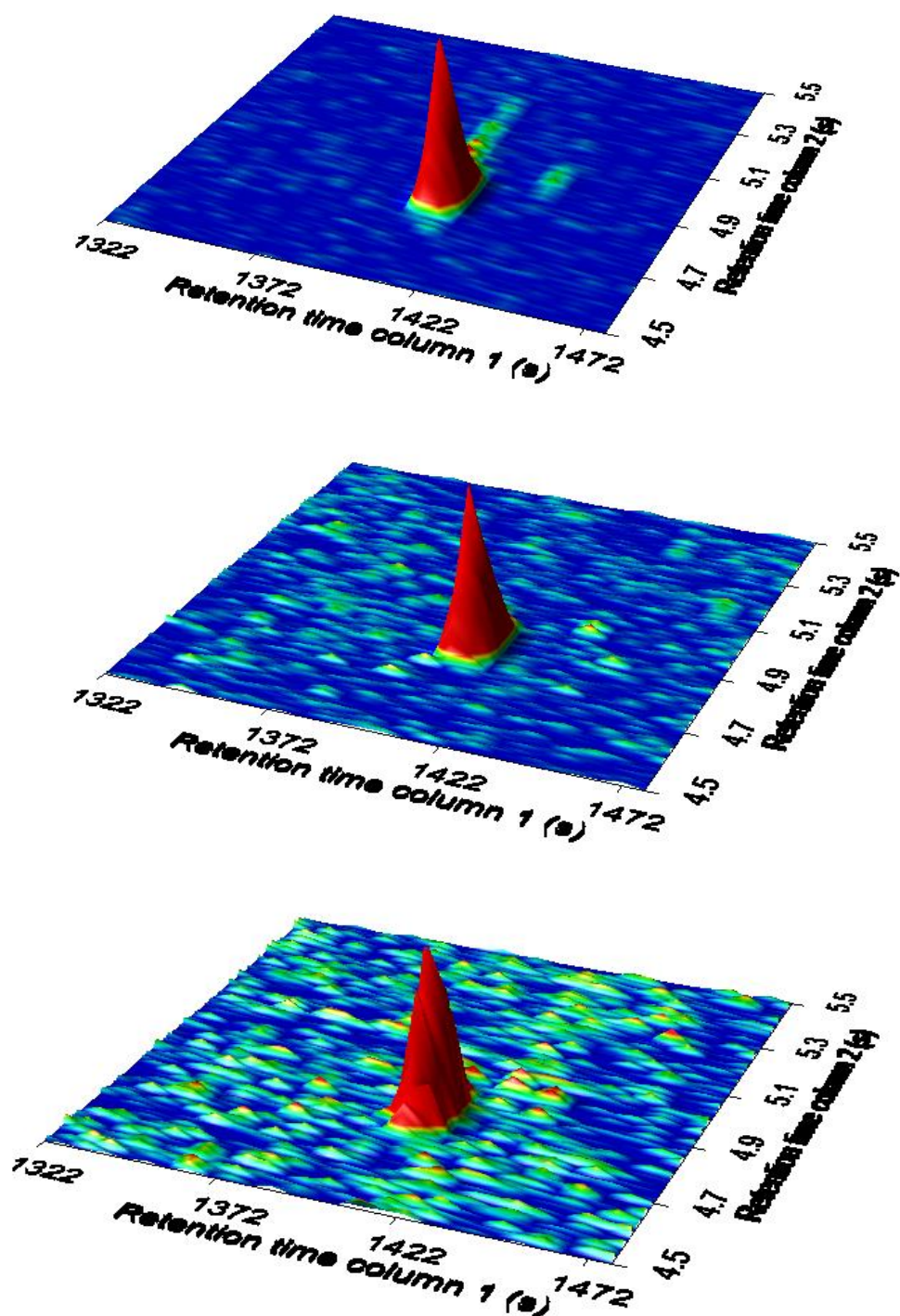


Figure 4.9. 2D surface plots illustrating the responses obtained for Xtren using its diagnostic fragmentation ion ($m/z = 174$). The peaks result from injections of 100, 50 and 25 pg of Xtren, respectively.

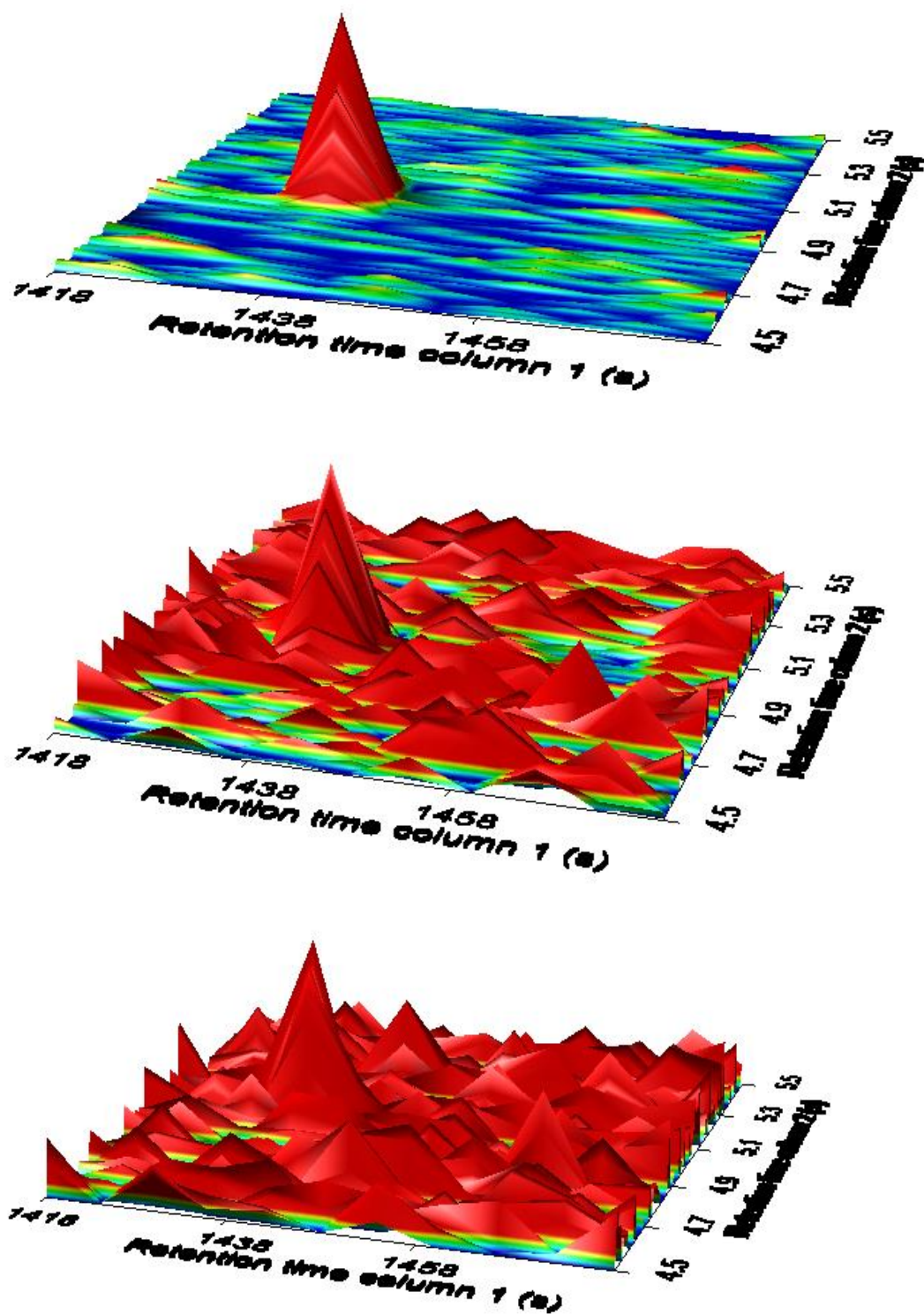


Figure 4.10. 2D surface plots illustrating the responses obtained for trenazone using its diagnostic fragmentation ion ($m/z = 135$). The peaks result from injections of 100, 50 and 25 pg of trenazone, respectively.

As shown earlier, epistane is well retained by both columns and elutes after both Xtren and trenalone. As can be seen in Figures 4.11, the epistane peak is clearly identifiable at 100 pg. In contrast, at 50 and 25 pg, whilst the elution region can be identified, the peak is not significantly above the background noise level.

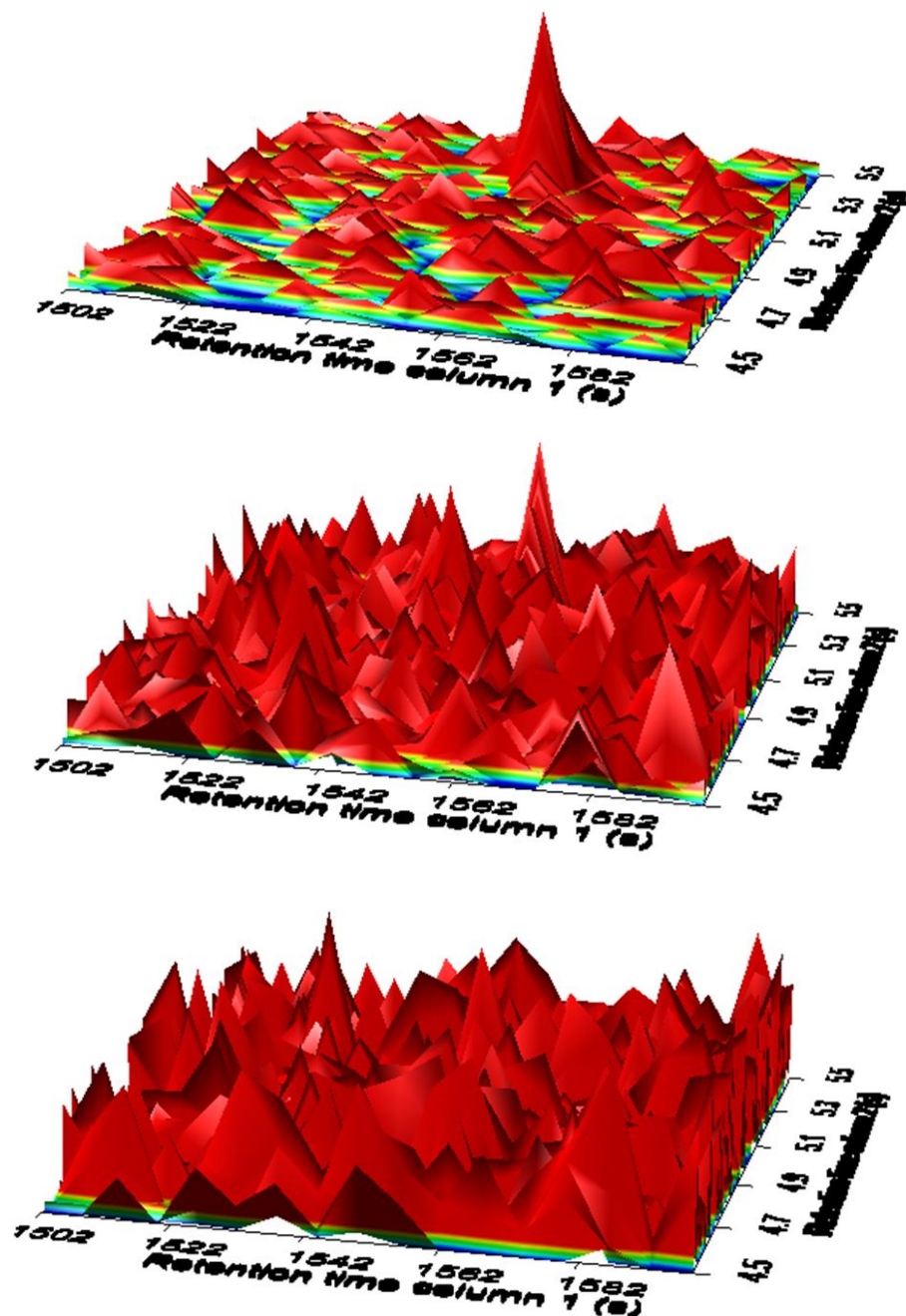


Figure 4.11. 2D surface plots illustrating the responses obtained for epistane, using its diagnostic fragmentation ion ($m/z = 255$), injected at 100, 50 and 25 pg on column, respectively.

Both Gestrinone [17] and THG [18] elute outside of the modulation window, taking more than 6 s to elute from the second column. The responses obtained for both gestrinone and THG can be seen in Figures 4.12. Gestrinone is detectable down to 100 pg, whereas THG can be clearly identified at 50 pg. Neither can be seen at 25 pg.

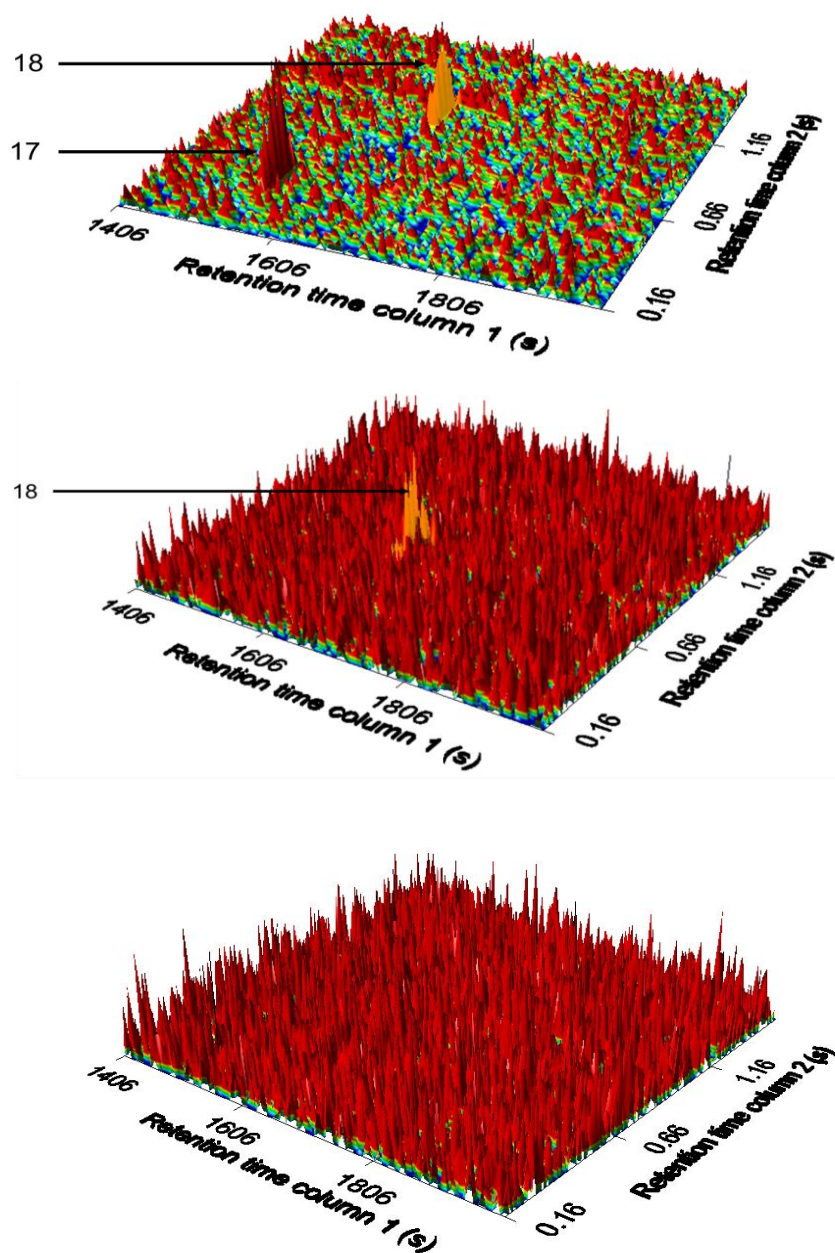


Figure 4.12 2D surface plots illustrating the responses obtained for gestrinone [17] and THG [18] using $m/z = 227$. The peaks result from injection of 100, 50 and 25 pg of each on column, respectively.

Utilising the proprietary instrument software, areas have been determined for all the steroid peaks identified in the previous tables with a signal to noise ratio of greater than three ($S/N > 3$) for their quantitation ion. These peak areas have then been plotted against the mass of steroid injected and used to determine the equations and the squared correlation coefficients for the linear least square fit over the mass range of 25 to 250 pg for the prohormones and over the mass range of 25 to 500 pg for the designer steroids.

The plots of the peak areas obtained for the quantitation ion for each of the steroids are provided in Figure A3.1 in Appendix A3. All the peak areas for the prohormones are shown to be linearly correlated with the mass injected in to the mass spectrometer, over the mass range of 25 to 250 pg. The regression equations and the squared correlation coefficients for each of the prohormones are provided in Table 4.6.

Table 4.6. The equations and the squared correlation coefficients for the linear least square fit corresponding to peak areas obtained for each of the prohormones, for injections over the mass range of 25 to 250 pg.

Steroid Number	Steroid Name	Regression Equation	R ²
1	5(10)-estrene-3b,17b-diol	$y = 28.013x + 44.024$	0.8503
2	4-estrene-3b,17b-diol	$y = 72.241x - 1843.6$	0.969
3	5(10)-estrene-3,17-dione	$y = 58.133x - 1104.1$	0.9599
4	4-androstene-3b,17b-diol	$y = 256.38x - 7032.6$	0.9822
5	DHEA	$y = 120x - 3257.6$	0.9502
6	5(6)-androstene-3b,17b-diol	$y = 84.616x - 2213.3$	0.9884
7	5a-androstane-3b,17b-diol	$y = 83.622x - 1958$	0.9644
8	5a-androstane-3,17-dione	$y = 125.2x - 3154.1$	0.9751
9	Nandrolone	$y = 245.4x + 1421$	0.8864
10	5(6)-androstene-3,17-dione	$y = 116.2x + 897.65$	0.8787
11	Testosterone	$y = 665.57x - 12489$	0.9781
12	1,4-androstadiene-3,17-dione	$y = 1830.2x - 32682$	0.9516

The squared correlation coefficients range between 0.8503 for 5(10)-estrene-3b,17b-diol and 0.9884 for 5(6)-androstene-3b,17b-diol. The plots for both these prohormones are provided for illustrative purposes in Figure 4.13.

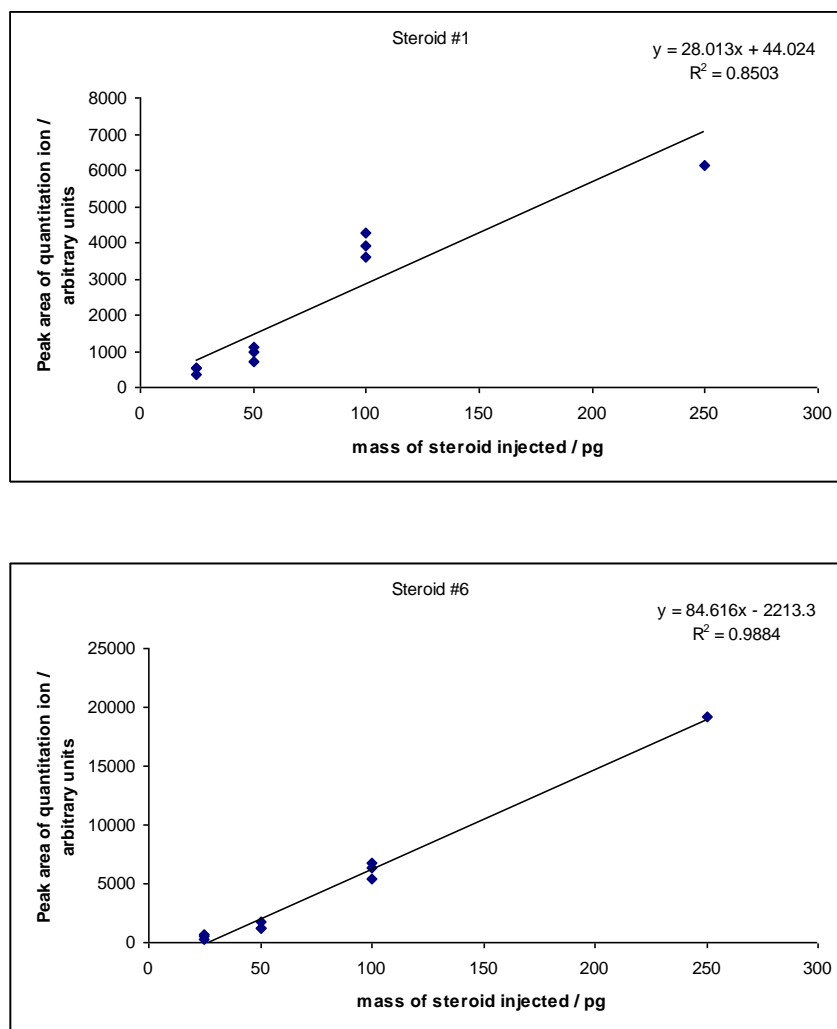


Figure 4.13. Plots of the peak areas obtained for 5(10)-androstene-3b,17b-diol [1] and 5(6)-androstene-3b,17b-diol [6], over the mass range of 25 to 250 pg.

The plots for the ratio of the peak area obtained for the quantitation ion for each of the prohormones versus the peak area obtained for the internal standard (methyltestosterone, m/z 91), over the mass range of 25 to 100 pg, are to be found in Figure A3.2 in Appendix A3. The regression equation and the squared correlation coefficients for the ratio of the peak areas of the prohormones relative to the internal standard are provided in Table 4.7.

Table 4.7. The equations and the squared correlation coefficients for the linear least square fit corresponding to the ratio of the peak areas obtained for each of the prohormones relative to the internal standard, for injections over the mass range of 25 to 100 pg.

Steroid Number	Steroid Name	Regression Equation	R ²
1	5(10)-estrene-3b,17b-diol	$y = 0.0028x - 0.037$	0.9031
2	4-estrene-3b,17b-diol	$y = 0.0027x + 0.007$	0.8952
3	5(10)-estrene-3,17-dione	$y = 0.0043x - 0.0874$	0.9608
4	4-androstene-3b,17b-diol	$y = 0.0122x - 0.1764$	0.9822
5	DHEA	$y = 0.0035x + 0.0533$	0.9385
6	5(6)-androstene-3b,17b-diol	$y = 0.0045x - 0.079$	0.9895
7	5a-androstane-3b,17b-diol	$y = 0.0029x + 0.036$	0.807
8	5a-androstane-3,17-dione	$y = 0.005x - 0.0057$	0.9921
9	Nandrolone	$y = 0.022x - 0.096$	0.969
10	5(6)-androstene-3,17-dione	$y = 0.0101x - 0.0084$	0.9683
11	Testosterone	$y = 0.0263x + 0.3471$	0.952
12	1,4-androstadiene-3,17-dione	$y = 0.0632x + 1.7405$	0.5973

The squared regression coefficient (R²) varies between 0.807 for 5a-androstane-3b,17b-diol and 0.9921 for 5a-androstane-3,17-dione, indicating that there is a good correlation between the mass of steroid injected and the ratio of the peak area of the steroid relative to the peak area of the internal standard. As expected, the squared regression coefficients (R²) has generally improved over the values obtained from straight area measurement, as this enables any slight variation in the experimental method to be accounted for. The obvious exception to this is the result obtained for 1,4-androstadiene-3,17-dione, which surprisingly

indicates that there is no significant correlation, with an R^2 of 0.5973. On studying the graph, shown in Figure 4.14, it appears that one of the data points at 100 pg is much lower than the other two, and may be producing the anomalous value. If this point were to be removed then the equation becomes $y = 0.0885x + 0.8556$ and $R^2 = 0.9285$, indicating that there is a good correlation with the mass of steroid injected. However, given the limited number of data points available (9), it is difficult to justify any statistically robust approach that would justify the removal of the data point. It is acknowledged that, in hind sight, this compound should have been re-analysed and that this result should be considered in any future study. However, it should also be noted that as a different approach is to be used for calculating the recovery of steroids using MIPS, then this anomalous result has no significant bearing on the rest of the chapter.

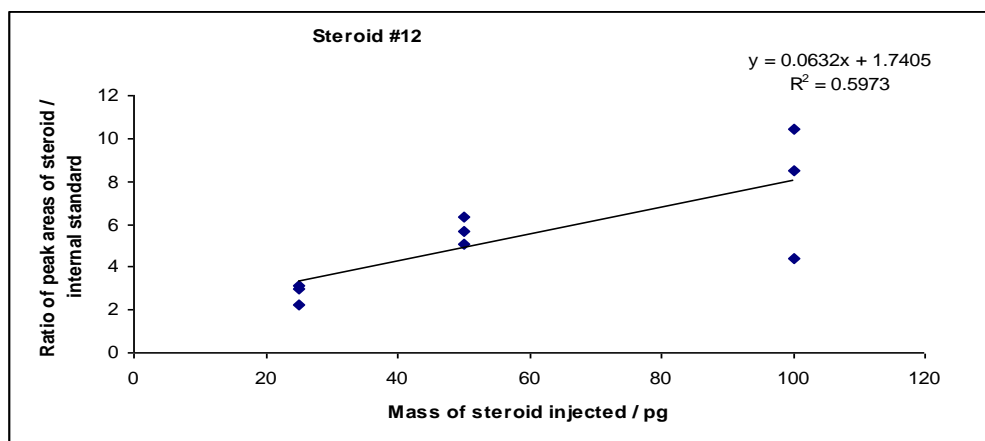


Figure 4.14. A plot of the ratio of the peak areas obtained for 1,4-androstadiene-3,17-dione [12] and the internal standard over the mass range of 25 to 100 pg.

Having established that the peak areas measured for the prohormones could be linearly correlated with the mass of steroid injected (with the exception of 1,4-androstadiene-3,17-dione), the attention turned to evaluating the peak areas for the designer steroids. Table 4.8, summarises the equations and squared correlation coefficients for the linear least square fit for the peak areas for injections over the mass range of 25 to 500 pg.

Table 4.8. The equations and the squared correlation coefficients for the linear least square fit corresponding to the peak areas obtained for each of the designer steroids, for injections over the mass range of 25 to 500 pg.

Steroid Number	Steroid Name	Regression Equation	R²
13	Epitestosterone	$y = 49.935x - 158.14$	0.9110
14	Xtren	$y = 405.06x + 1348.8$	0.9604
15	Trenazone	$y = 78.661x - 631.98$	0.9319
16	Epistane	$y = 19.691x - 699.01$	0.9845
17	Gestrinone	$y = 14.904x + 288.69$	0.9086
18	THG	$y = 17.612x - 256.14$	0.9171

The squared correlation coefficients range between 0.9086 for gestrinone and 0.9845 for epistane, although it should be noted that due to higher detection limits ($S/N > 3$) there are fewer data points than for the prohormones. As previously reported, epistane had only one peak above the limit of detection at 50 pg and no peaks were attributable at 25 pg. Gestrinone had only two peaks above the limit of detection at 50 pg and no peaks were attributable at 25 pg whereas THG had no peaks attributable above the limit of detection, at 25 pg.

The linearity plots for the designer steroids can be found below, in Figures 4.15 and 4.16 and also in Figure A3.3 in Appendix A3.

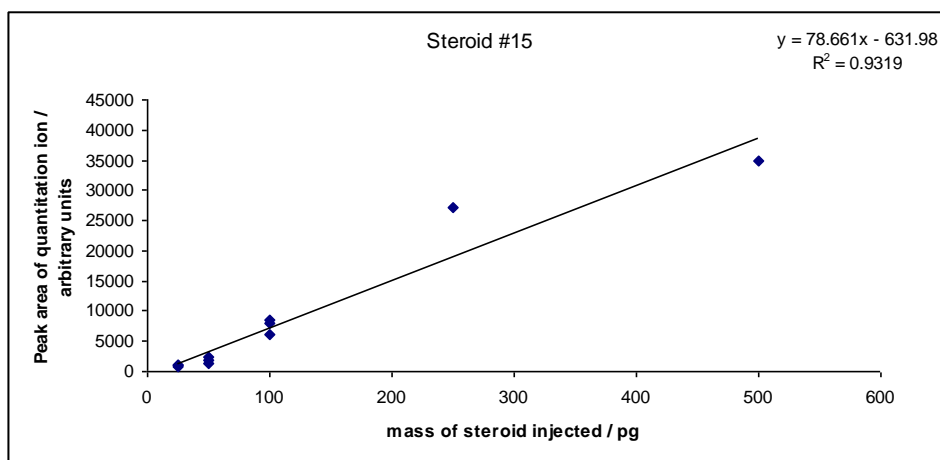
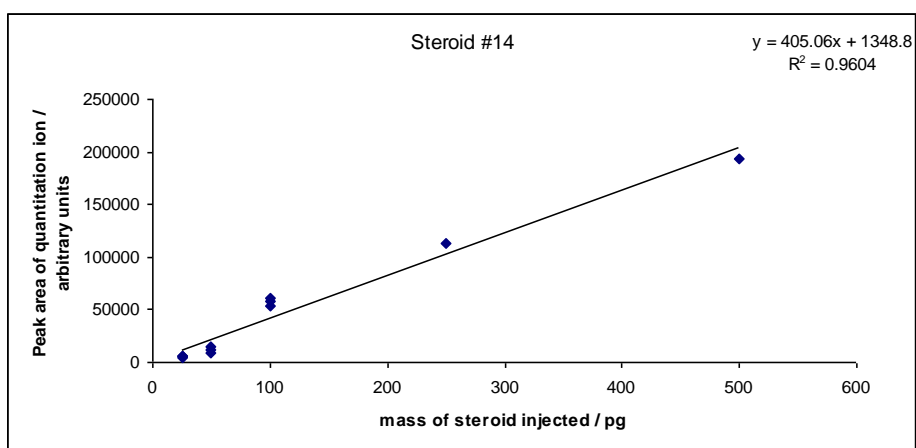
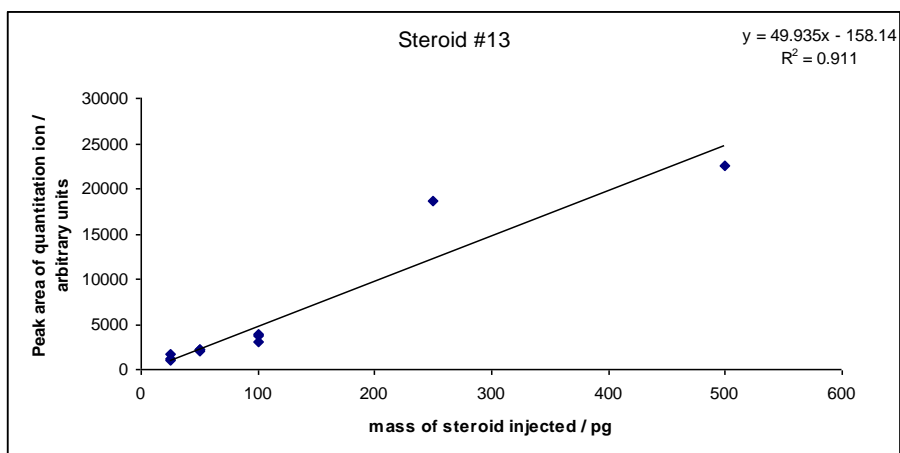


Figure 4.15 Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epitestosterone [13], Xtren [14] and trenazone [15], over the mass range 25 to 500 pg.

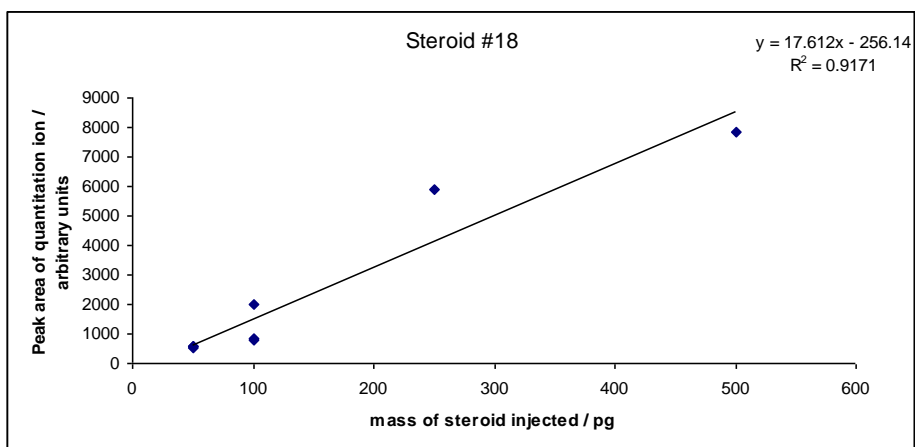
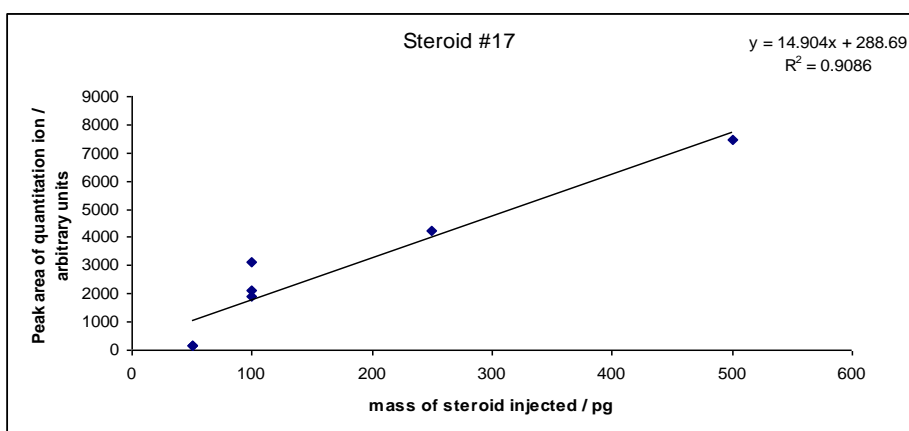
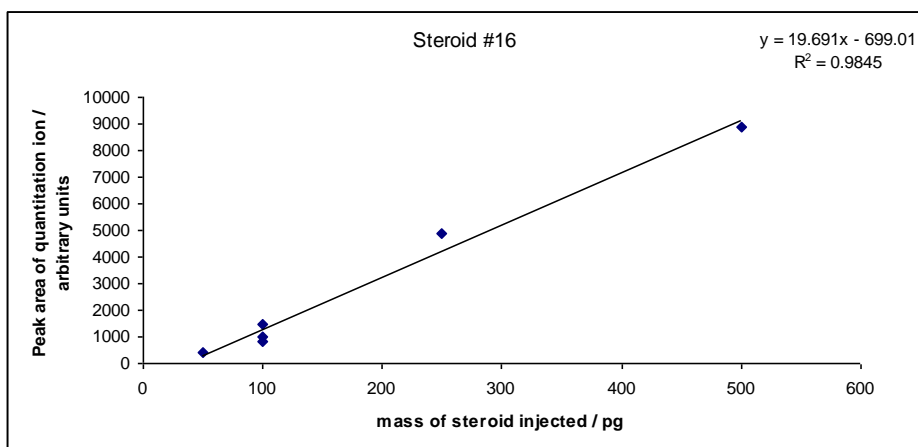


Figure 4.16. Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epistane [16], gestrinone [17] & THG [18], over the mass range 25 to 500 pg.

The linearity plots for two of the designer steroids (epitestosterone and trenazone) may be being influenced by the single data point at 250 pg. As previously stated, with the limited number of data points there is no statistically robust reason or any apparent systematic experimental error to justify removing these values. The squared correlation coefficients for these two compounds are also acceptable. Most tests for outliers (e.g. Grubb Test or Dixon Q Test) assume a Gaussian distribution, requiring more data than available. The experiment would have been improved if triplicate measurements had been taken at 250 and 500 pg and this approach will be applied in future. As with 1,4-androstadiene-3,17-dione, it should also be noted that a different approach is to be used for calculating the recovery of steroids using MIPS. These results therefore have no significant bearing on the rest of the chapter.

A detailed summary of the conclusions of this part of the study will be provided in the conclusion section of this chapter (Section 4.3). For now it is suffice to say that based on the above findings it was decided to evaluate the molecular imprinted polymer method, utilising urine standards containing designer steroids (spiked at the 10 ng/mL level) in the presence of prohormones (spiked at the 2-10 ng/mL level) and the internal standard (methyltestosterone, spiked at the 500 ng/mL level).

4.2.2. Analysis of designer steroids following extraction from synthetic urine using molecularly imprinted polymers.

Spiked urine standards were prepared as previously described in Section 2.2.11. and the subsequent extractions were performed as per the method described in the same section. Three cartridges of both M6 and M5, as used in the Chapter 3 study, were re-used following washing with 1 mL of water, acetonitrile and then methanol.

It should also be noted that the starting temperature of the gas chromatography ramp was increased slightly from 50 °C in the above standard study to 65 °C in this study. This step was taken to reduce the overall duty cycle for the cooling of the primary and secondary ovens between runs. It was not thought to have a significant bearing on the chromatography of the steroids, except that they would elute ~1 minute earlier than previously (given the 15 °C/min ramp rate).

Figure 4.17 to 4.20 provide representative surface plots for each of the designer steroids obtained by extraction, by molecular imprinted polymers (M6 or M5), from 1 mL of synthetic urine (spiked at 10 ng/mL). Except for epistane, each of the designer steroids is clearly detectable at the 10 ng/mL level in the reconstituted eluent from each of the M6 cartridges.

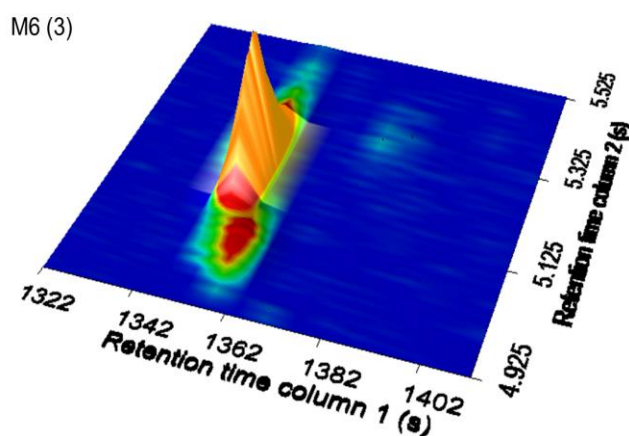
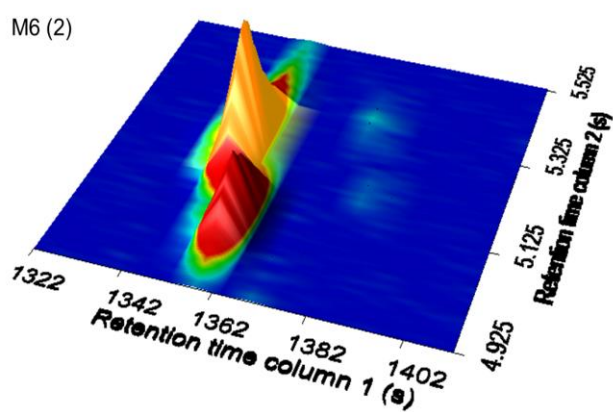
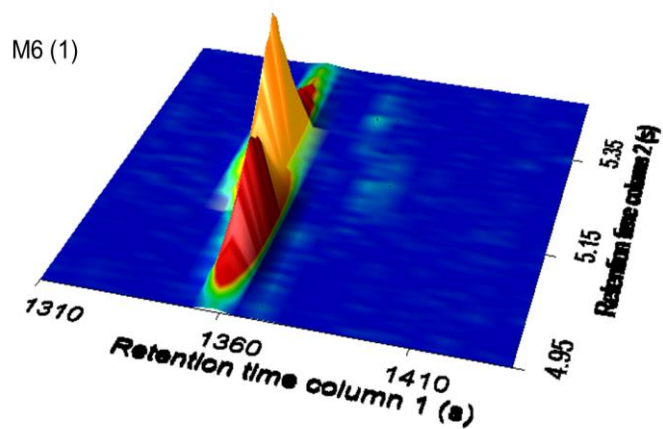


Figure 4.17. 2D surface plots obtained for Xtren [14], using its diagnostic fragmentation ion ($m/z = 174$). The peaks result from the extraction of the designer steroid from 1 mL of synthetic urine, spiked at the 10 ng/mL level, using the molecular imprinted polymer, M6. The extractions were conducted in triplicate on three separate cartridges. Virtually identical plots were obtained using the M5 cartridges.

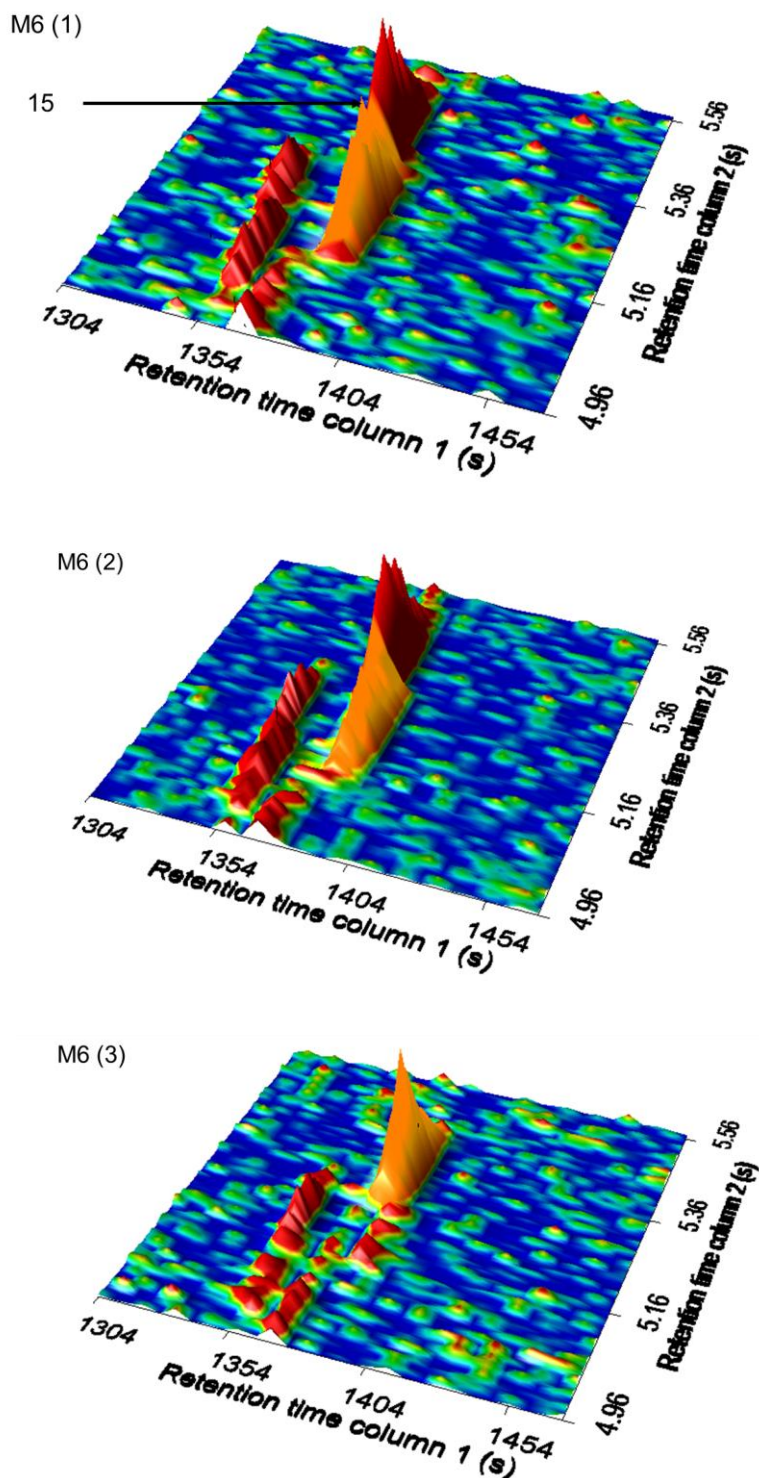


Figure 4.18. 2D surface plots obtained for trenazone [15], using its diagnostic fragmentation ion ($m/z = 135$). The peaks result from the extraction of the designer steroid from 1 mL of synthetic urine, spiked at the 10 ng/mL level, using the molecular imprinted polymer, M6. The extractions were conducted in triplicate on three separate cartridges. Virtually identical plots were obtained using the M5 cartridges.

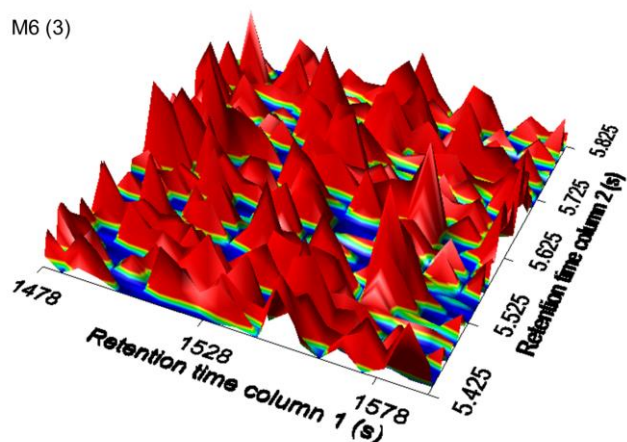
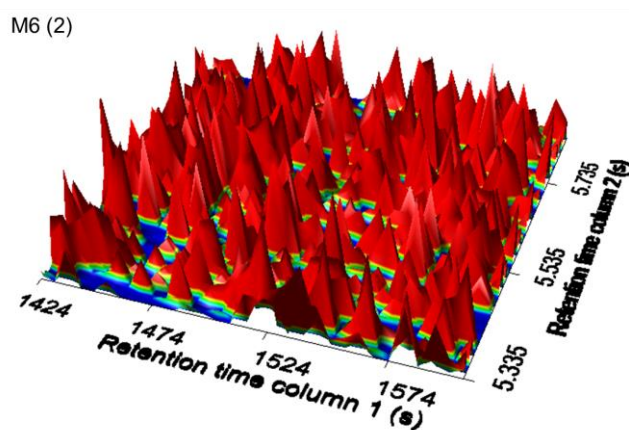
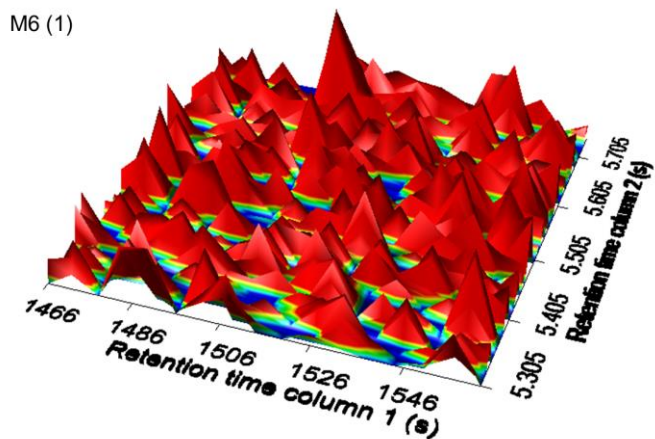


Figure 4.19. 2D surface plots obtained for epistane [16], using its diagnostic fragmentation ion ($m/z = 255$). The peaks result from the extraction of the designer steroid from 1 mL of synthetic urine, spiked at the 10 ng/mL level, using the molecular imprinted polymer, M6. The extractions were conducted in triplicate on three separate cartridges. Virtually identical plots were obtained using the M5 cartridges.

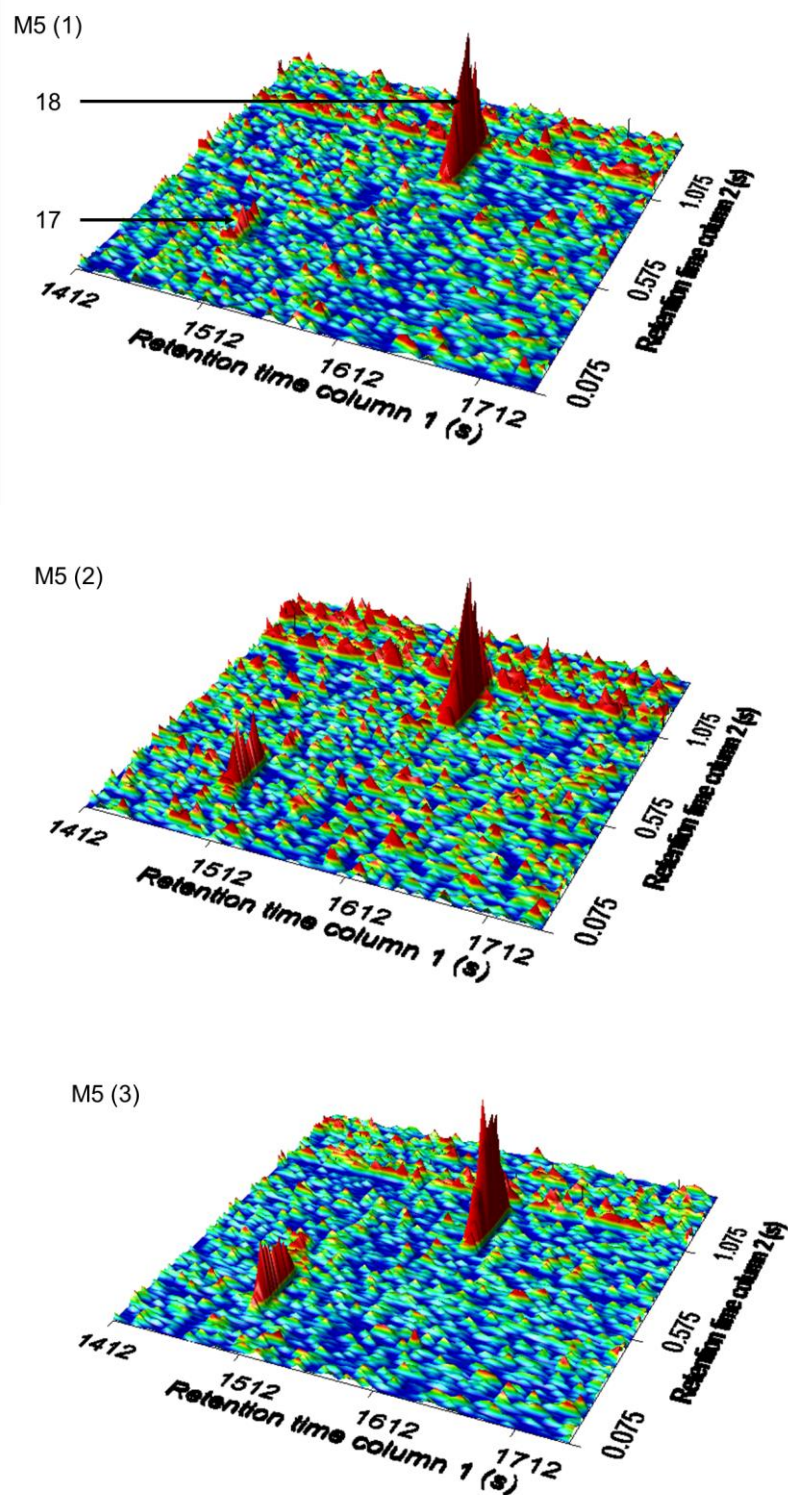


Figure 4.20. 2D surface plots obtained for gestrinone [17] and THG [18], using their diagnostic fragmentation ion ($m/z = 227$). The peaks result from the extraction of the designer steroid from 1 mL of synthetic urine, spiked at the 10 ng/mL level, using the molecular imprinted polymer, M5. The extractions were conducted in triplicate on three separate cartridges. Virtually identical plots were obtained using the M6 cartridges (except for M6(3)).

Table 4.9 summarises the percentage recovery obtained on each of the M6 molecular imprinted polymer cartridges, for each of the designer steroids. These values were derived by dividing the peak area for the quantitation ion for each steroid by the peak area determined for the internal standard in each sample. This ratio was then further divided by the average of the same ratios determined for the two bracketed standards. Multiplying by 100 gave the percentage recovery.

Table 4.9. The percentage recovery determined for each of the designer steroids in each of the M6 molecular imprinted polymers cartridges, relative to the average areas obtained from bracketed standards. 1 mL of synthetic urine spiked at 10 ng/mL.

Steroid Number	Steroid Name	Recovery M6 (1) (%)	Recovery M6 (2) (%)	Recovery M6 (3) (%)	Average Recovery (%)	Recovery Standard Deviation (%)
13	Epitestosterone	30.1	15.5	33.0	26.2	9.4
14	Xtren	35.3	35.1	38.2	36.2	1.7
15	Trenazone	30.2	16.5	20.6	22.4	7.0
16	Epistane	0	0	0	0	0
17	Gestrinone	1.0	1.1	7.4	3.2	3.6
18	THG	145.8	72.6	100.7	106.3	36.9

Table 4.10 summarises the percentage recovery obtained on each of the M5 molecular imprinted polymer cartridges, for each of the designer steroids. The values were derived in the same way as for M6.

Table 4.10. The percentage recovery determined for each of the designer steroids in each of the M5 molecular imprinted polymers cartridge, relative on the average areas obtained from bracketed standards. 1 mL of synthetic urine spiked at 10 ng/mL.

Steroid Number	Steroid Name	Recovery M5 (1) (%)	Recovery M5 (2) (%)	Recovery M5 (3) (%)	Average Recovery (%)	Recovery Standard Deviation (%)
13	Epitestosterone	31.3	23.7	15.0	23.4	8.2
14	Xtren	32.4	37.7	57.7	42.6	13.3
15	Trenazone	31.0	32.0	26.6	29.9	2.9
16	Epistane	0	0	0	0	0
17	Gestrinone	1.4	5.2	6.7	4.4	2.7
18	THG	165.0	130.4	98.4	131.3	33.3

From the above tables we are able to conclude that the average percentage recoveries determined for the M5 cartridges are highly consistent with those determined for M6. In addition, the recoveries of individual designer steroids are highly reproducible between the replicate cartridges.

It is also clear, with the exception of THG, that the percentage recovery of each of the designer steroids is much lower than was achieved for the prohormones.

THG had the highest recovery on all six of the MIPs cartridges, with an average percentage recovery of 106.3 % on M6 and 131.3 % on M5 cartridges, respectively.

Gestrinone had very low recovery on all six of the MIPs cartridges, with an average percentage recovery of 3.2 % on the M6 cartridges and 4.4 % on the M5 cartridges, respectively.

Suprisingly, the average percentage recovery of epitestosterone was only 26.2 % on M6 and 23.4 % on M5. This would not have been expected in terms of its similarity to the testosterone molecule; however, the very close chromatographic proximity (co-elution) to testosterone made it difficult to determine accurate peak areas.

Xtren and trenazone had very similar recoveries to epitestosterone, with the average percentage recovery of Xtren being 36.2 % on M6 and 42.6 % on M5 and the average percentage recovery of trenazone being 22.4 % on M6 and 29.9 % on M5, respectively.

The failure to detect epistane in any of the cartridges, despite having been detected at lower concentration level in the previous validation study would indicate that the unique conformation of epistane, with its bridging sulphur group, means that it is either not being extracted from the urine or it is being retained within the MIPs. The former is more likely and is an additional piece of evidence that the MIPs material has developed cavities that were templated around the testosterone molecule.

It is not clear if the presence of the full cohort of the prohormones in the urine samples, ranging in concentration from 2-10 ng/mL, has significantly influenced the performance of the MIPs for the designer steroids. It may be possible that there is competition for the cavities and that the prohormones are being selectively trapped. There is no clear evidence of this being the case and therefore this would need to be evaluated in further studies.

Despite the lower recoveries all the designer steroids, all except epistane, are clearly visible in the 2D surface plots provided in Figures 4.16 to 4.19.

At this stage, the performance of the cartridges are acceptable, despite the low percentage recovery. They are very reproducible and have been shown to be viable for the detection of most of the designer steroids present in synthetic urine at 10 ng/mL. Further studies will be necessary to determine batch-to-batch variability and how the cartridges perform if they are re-used and if they are used on urine samples at lower concentrations.

4.3. Conclusions

In addition to naming specific banned substances, the WADA Prohibited Lists also carry the caveat of “other substances with a similar chemical structure or similar biological effect(s) are also prohibited”. Designer steroids fit firmly into this category. With derivatisation methods shown to be highly compound specific, and other sensitive tests developed for LC/MS (MS) also being highly specific, there is no suitable “family-wide” protocol available for the screening of known, and unknown, prohormones or designer steroids. Therefore, by targeting the native state of the steroids and utilising the power of comprehensive gas chromatography and molecular imprinted polymers (MIPs) we have developed a screening method for a family of prohormones and designer steroids that share the testosterone nucleus.

Based on the findings of the above studies to evaluate the suitability of the previously developed comprehensive chromatography method to resolve, detect and quantify a cohort of designer steroids in the presence of the original prohormones, it can be concluded that:

- i. Most of the cohort of six designer steroids elute in different regions to that of the prohormones;
- ii. The exception is epitestosterone, the 17 alpha-epimer of testosterone. As anticipated, epitestosterone and testosterone partially co-elute making them difficult to quantify as they also have the same fragmentation pattern;
- iii. Xtren and trenazone, having a similar estradiene configuration, elute in a similar area and are retained longer than the prohormones;
- iv. Epistane, with its bridging sulphur group, is retained on both columns for longer than either Xtren or trenazone. It also elutes later than gestrinone in the first dimension yet interestingly it elutes earlier on the second dimension column, which has a greater level of aromatic substituents;
- v. Gestrinone and THG, being estratrienes have the longest elution times on the second dimension, taking longer to elute than the current modulation time allows. As a result of this 'wrap around' they are found in their own distinct region of the surface plot. Their greater retention is due to the fact that their three conjugated carbon-carbon double bonds and the ketone group at the 3-position have the greatest interaction with the aromatic substituents in the stationary phases;
- vi. Retention times are consistent in the first dimension; however, due to modulation, small changes in the second dimension retention time are possible. The one exception is gestrinone which elutes from the second dimension column at around the time the modulator switches, which can result in significant changes in its second dimension retention time. This does not tend to cause an issue as the full mass range of ions (m/z 50 to 521) are available and the compound can be identified by comparison of its fragmentation pattern with library values;

- vii. The use of underivatised steroids produces high spectral similarity between the library fragmentation patterns and those found for the steroids in the samples, as it prevents ambiguous identification due to derivatisation issue. As expected, the similarity increases when the concentrations of the steroid increases, as the background noise becomes less significant;
- viii. The areas measured for the individual quantitation ions for each of the prohormones and designer steroids has been shown to be correlated with the amount of steroid injected;
- ix. Not all of the designer steroids were detectable above background noise, in all the standard solutions injected:
 - a. Xtren and trenazone were detectable at 25 pg
 - b. Epistane was only detectable down to 100 pg
 - c. THG was detectable at 50 pg but not at 25 pg
 - d. Gestrinone was only detectable down to 100 pg

Based on the extraction method employed throughout the thesis, 0.25 pg on column would be equivalent to a concentration of 0.5 ng/mL in urine; similarly 50 pg would be equivalent to 1.0 ng/mL in urine and 100 pg equivalent to 2.0 ng/mL. Once again the caveat is that these figures are for unconjugated steroid standards and they were not extracted from synthetic urine;

- x) The average percentage recovery of the designer steroids, on triplicate M6 cartridges, ranged between 0 - 106.3 % when extracted from 1 mL of synthetic urine spiked at 10 ng/mL;

- xi) The average percentage recovery of the designer steroids, on triplicate M5 cartridges, ranged between 0 - 131.3 % when extracted from 1 mL of synthetic urine spiked at 10 ng/mL.
- a. THG had the highest recovery on all six of the MIPs cartridges, with an average percentage recovery of 106.3 % on M6 and 131.3 % on M5, respectively;
 - b. Gestrinone had very low recovery on all six of the MIPs cartridges, with an average percentage recovery of 3.2 % on the M6 cartridges and 4.4 % on the M5 cartridges, respectively;
 - c. Surprisingly, the average percentage recovery of epitestosterone was only 26.2 % on M6 and 23.4 % on M5. This would not have been expected in terms of its similarity to the testosterone molecule; however, the very close chromatographic proximity (co-elution) to testosterone made it difficult to determine accurate peak areas;
 - d. Xtren and trenazone had very similar recoveries to epitestosterone, with the average percentage recovery of Xtren being 36.2 % on M6 and 42.6 % on M5 and the average percentage recovery of trenazone being 22.4 % on M6 and 29.9 % on M5, respectively;
 - e. Epistane was not detected in any of the eluents from the MIPs cartridges, suggesting that was not extracted from the urine. The most likely explanation is that this is due to it not sharing the same conformation as

testosterone and therefore it does not bind efficiently in the templated cavities;

- xii) It is not clear if the presence of the prohormones significantly impacted the performance of the method and this should be explored further in the future;
- xiii) Further evaluation will be required to determine if there is any significant influence on the performance of the MIPs when analysing real, as opposed to synthetic urine.

Whilst the average percentage recoveries for all the designer steroids, except for THG, were lower than those obtained for the prohormones, the recoveries were consistent and reproducible. Further studies will be necessary to determine batch-to-batch variability and to observe how the cartridges perform if they are re-used, and also to assess the potential influence in moving from synthetic to human urine samples and towards conjugated steroids. The availability of conjugated steroids was beyond the scope of this thesis.

It is clear that not all the designer steroids are compatible with the method developed and further work is required to fully characterise their performance for a range of compounds. The screening capability is determined by both the response factor of the designer steroid in the GCxGC/MS and the recovery from the MIPs. Both epistane and gestrinone have very low recoveries from the MIPs and have the highest detection levels (100 pg on column for standards, equivalence of 2 ng/mL in urine). MIPs templated with testosterone are not applicable to the extraction of epistane and it is highly unlikely to be applicable in sports testing for gestrinone. Xtren, trenazone and THG have acceptable recoveries and better response factors (equivalence of 0.5 ng/mL, 0.5 ng/mL and 1.0 ng/mL, respectively). When taking this and the percentage recovery by the MIPs in to consideration, then they should be able to be detected at the 5 ng/mL level in urine. However, as stated previously, this test is

currently for unconjugated steroids and therefore will require the urine sample to be processed, prior to extraction, especially with the lower MRPL levels now required by WADA since 2012.

Despite this draw back, the MIPs method has been shown to remove matrix components and the power of comprehensive chromatography coupled to TOFMS, reduces the probability of co-elution of the target compounds with system blanks, matrix compounds, masking agents, reducing the likelihood of obtaining false positive and false negative results. The resolving power of comprehensive gas chromatography coupled with the high acquisition rate of the TOFMS also makes it an ideal tool for the screening of unknown compounds. Capturing the full mass spectrum also allows for retrospective analysis, should new compounds emerge and co-elute.

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NB References marked with *** at the end are non-peer reviewed articles on Designer Steroids

- 5. A pilot study evaluating the potential of aptamer coated magnetic beads for the extraction of estradiol prior to analysis by mass spectrometry**

5.1. Introduction

The previous chapters have shown that molecular imprinted polymers (MIPS), when coupled with comprehensive gas chromatography and mass spectrometry, can enhance the existing portfolio of techniques currently available to the analyst. However, the analysis of steroids is not limited to the sports testing arena, clinical applications also have similar requirements. In addition, the extraction of steroids from biological matrices is not limited to the application of molecular imprinted polymers or solid phase extraction. Other analytical approaches, such as magnetic beads coated with aptamers, may also provide an alternative, as summarised in Section 1.2.6.

This chapter presents a pilot study exploring the potential of an aptamer, coated on to magnetic beads, as a clean-up and extraction procedure for estradiol, prior to analysis by mass spectrometry. This work actually pre-dated the MIPS studies and the selection of estradiol as the target steroid was driven by:

- i) the lack of a suitable aptamer for testosterone,
- ii) estradiol is a compound with significant clinical relevance.

Estradiol (E2) is a small molecule, containing just 18 carbon atoms and has a molecular weight of 272.4 Da. In mammals its synthesis occurs mainly in the ovaries; smaller amounts are synthesized by the adrenal cortex and by the testes. The development of secondary sexual characteristics in women is triggered by estradiol production, produced by the corpus luteum. Estradiol levels vary according to age and menstrual cycle. During the menstrual cycle, in pre-menopausal women, E2 serum levels will vary over a range from 41 to 272 pg/mL, and decrease to about 4–14 pg/mL after the menopause.¹

A determination of the amount of E2 in a biological matrix is often needed in investigative and clinical analysis, for example: during pregnancy, amenorrhea (including menstrual disorders), estrogen-secreting tumours, testicular function and gonadal agenesis. It has also been shown that elevated amounts of estradiol, or its metabolites, can activate the rapid growth of cancer cells.² Gynecomastia is linked to oestrogens formed in the adipose tissue and can cause obesity in men.³ Due to the strong link between estradiol and endocrine and gynaecological diseases, its monitoring and quantification remains an important aspect in clinical diagnosis.²⁻³

Given the low concentration of E2 in post-menopausal women and the high concentration levels of a large number of structurally related steroids present in the sample, the accuracy of E2 measurements can be affected. Assays developed for estradiol therefore require high sensitivity and selectivity.

5.2. Results and Discussion

The aptamer was bound to the magnetic beads using the materials and apparatus described in Sections 2.4.1 and 2.4.2 and the method reported in Section 2.4.7, respectively. The binding process was confirmed by measuring a drop in the UV absorbance, at a wavelength of 260 nm, after exposure to the beads.

The scanning electron microscope images in Figure 5.1, confirm that the spherical, super paramagnetic beads (Dynabeads M-280, coated with streptavidin) provided a consistent and uniform surface for conjugation with the chosen DNA aptamer (5-Biotin GCTTCCAGCTTATTGAATTACACGCAGAGGGTAGCGGCTCTGCGCATTCAATTG CTGCGCGCTGAAGCGCGGAAGC-3). During the study, the beads rapidly separated from the rest of the sample on the application of an external magnetic field, allowing the

matrix to be easily decanted, and they fully returned to solution when the external magnet was removed. This confirmed the manufacturer's claim that the beads would exhibit no residual magnetism once removed from the magnetic field.⁴

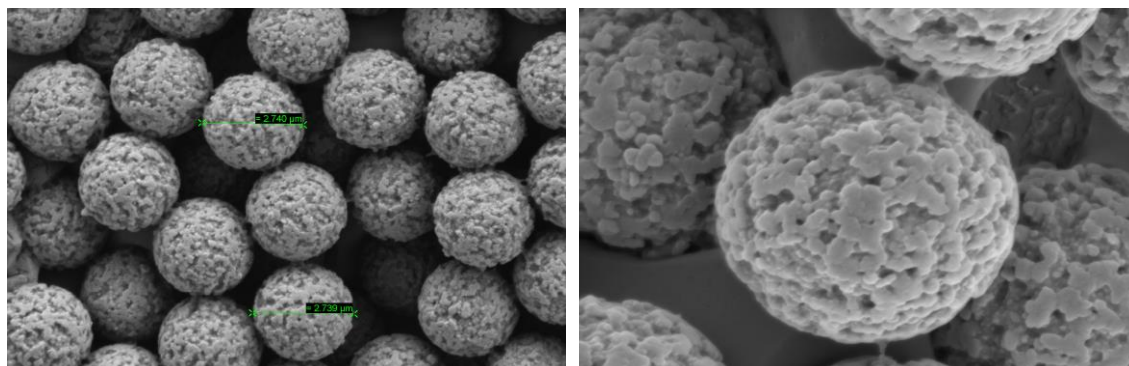


Figure 5.1. Scanning electron microscope images showing the aptamer attached to the magnetic beads following implementation of the binding procedure described in Section 2.4.3. The right hand image is a X3 magnification of the left hand image.

5.2.1. Evaluating the GC/MS response to different quantities of derivatised estradiol present in standard solutions

Prior to the evaluation of the aptamer performance, the response of the GC/MS to a range of derivatised estradiol (E2) standards was studied using the parameters described in Section 2.3.4. The derivatised estradiol standards were prepared as described in Section 2.3.3.

Estradiol, following derivatisation with MTBSTFA (1% TBDMCS), was shown to elute at 10.18 minutes and its identity was further confirmed using its full fragmentation pattern and a secondary ion (m/z 500). As shown in Figure 5.2, the peak areas are linear over the range 14,286 - 0.143 $\mu\text{g/mL}$, with a squared coefficient of regression of 0.9977. A spurious data point at 14.3 $\mu\text{g/mL}$ has been removed, as the area measured was of the same

magnitude as the value determined at 143 µg/mL, suggesting an error in the preparation of that solution.

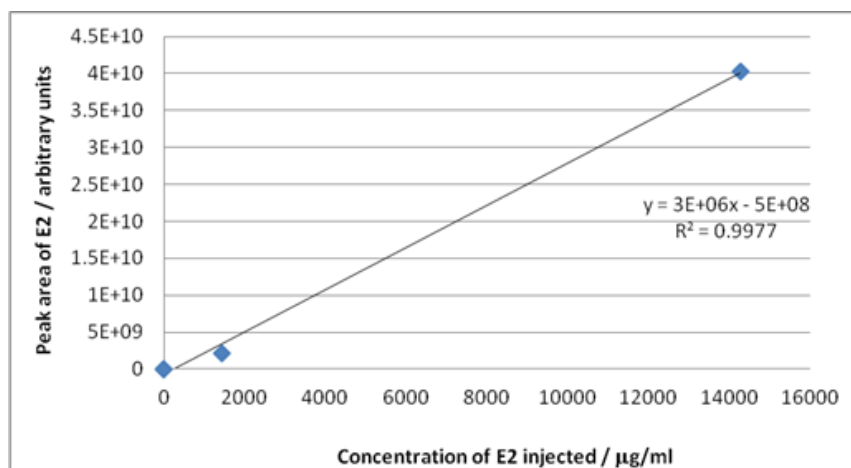


Figure 5.2. Showing the peak areas measured for serial dilutions of derivatised estradiol standards in methanol, from 1 µL injections over the concentration range of 14,286-0.143 µg/mL.

With a 1 µL splitless injection utilised throughout, this equates to between 14.3 µg and 0.143 ng of estradiol being injected in to the mass spectrometer. The signal to noise determined for the lowest concentration injection was 104, suggesting that the limit of quantitation (LOQ) achievable by GC-MS, by this method is in the tens of pg of estradiol.

When the above data was re-plotted over the reduced concentration range of 1,429-0.143 µg/mL (equating to injections of between 1.43 µg and 143 pg of estradiol) a slightly lower slope was obtained ($y = 1E+6x + 4E+7$) with a squared correlation coefficient of 0.9962.

5.2.2. Evaluating the binding efficiencies and the recovery of estradiol from the aptamer coated magnetic beads by GC/MS

Samples were prepared for analysis as described in Section 2.4.9. Analysis of the Hormone Binding Buffer, before and after addition to the aptamer coated magnetic beads, showed

that estradiol was not detectable above background noise in the second solution, suggesting that it had been efficiently trapped. The GC/MS trace obtained from the original solution is shown in Figure 5.3; whereas, the chromatogram obtained for the solution after vortexing with the aptamer is provided in Figure 5.4.

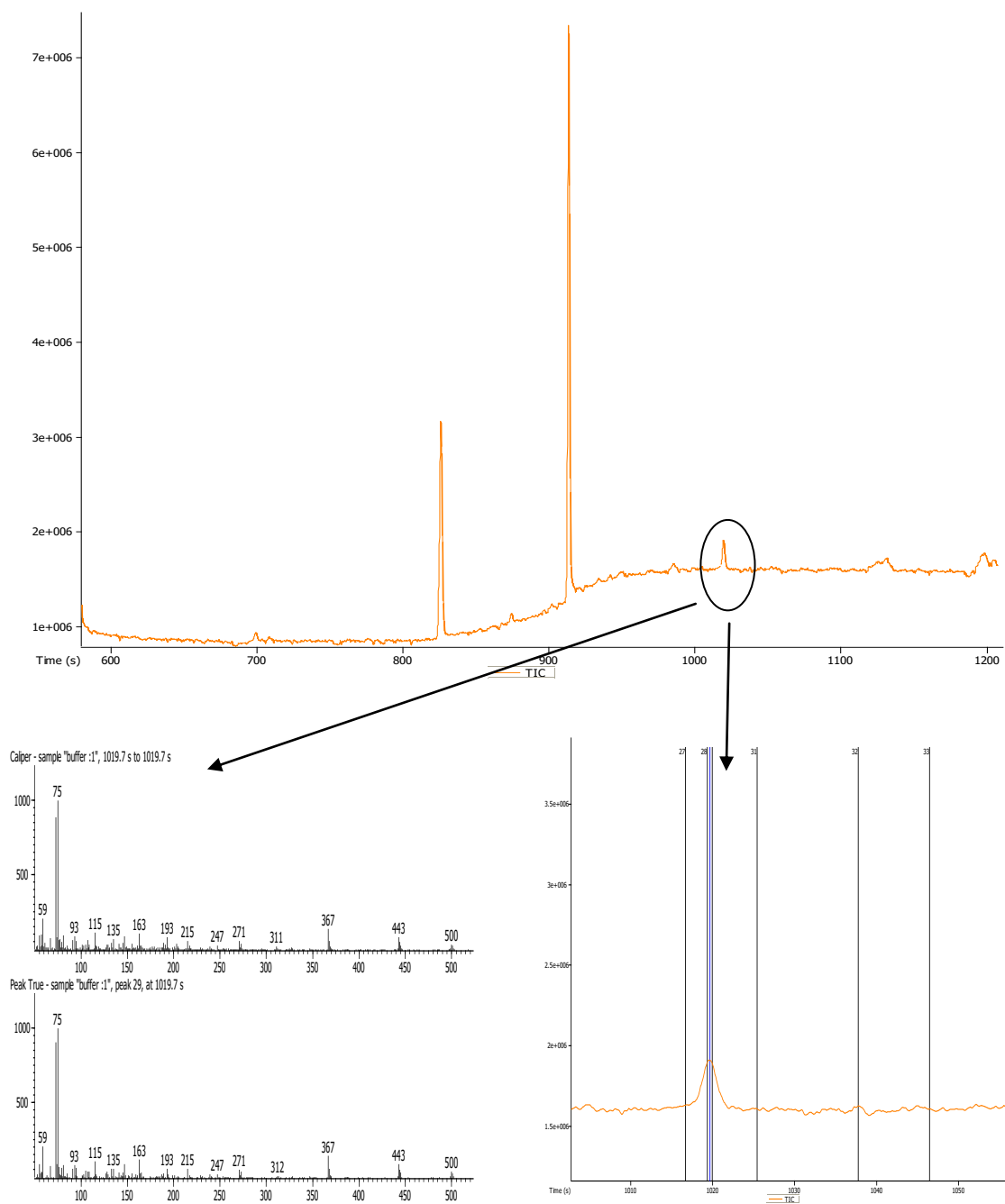


Figure 5.3. Chromatograms and mass fragmentation patterns showing the peak obtained for estradiol from the original Hormone Binding Buffer.

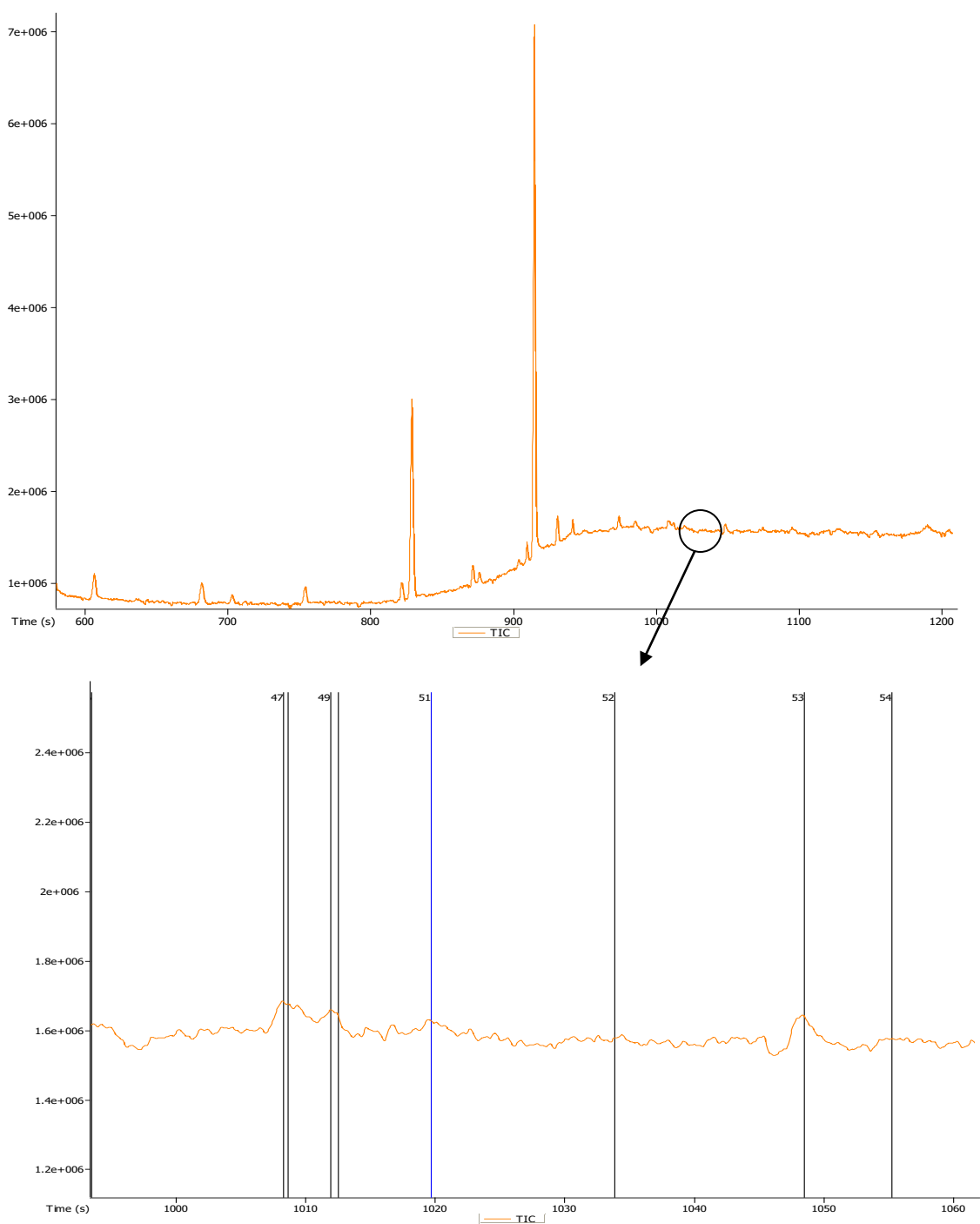


Figure 5.4 *Showing the chromatograms obtained for the Hormone Binding Buffer after vortexing with the aptamer coated magnetic beads. Estradiol was not detected above the background noise.*

Whilst the trapping of estradiol on to the aptamer appears efficient, the eventual percentage recovery of estradiol in the original Elution Buffer (40 mM Tris-HCl, 10 mM (EDTA), 3.5

mM urea), was found to be only 31.2 %. A comparison of the peak areas obtained for both solutions, processed in the same manner, is provided in Figure 5.5.

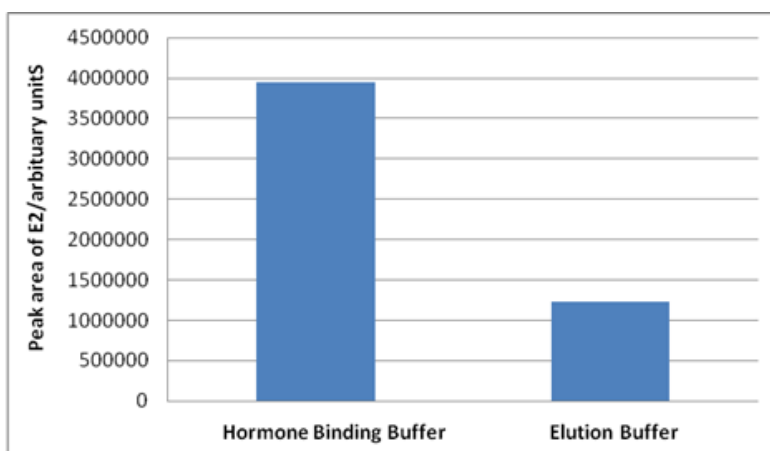


Figure 5.5. A comparison of the peak areas determined for estradiol in the Hormone Binding Buffer, as added to the aptamer coated beads, and in the original Elution Buffer supernatant.

A much higher recovery of estradiol (53.8 %) was obtained when the original Elution Buffer solution was replaced with 10 % sodium dodecyl sulphate (SDS). This higher recovery with SDS is illustrated in Figure 5.6 and as a result, SDS was deemed a more suitable elution buffer for estradiol, when analysed in its derivatised form by GC/MS.

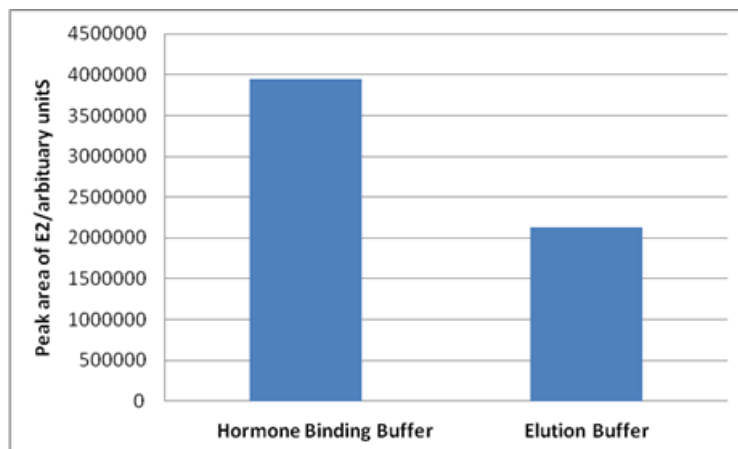


Figure 5.6. A comparison of the peak areas obtained for estradiol in the Hormone Binding Buffer added to the beads, and that obtained in the SDS Elution Buffer supernatant.

It should be noted that the pictorial representation of the method in Figure 2.1 of Chapter 2, for the selective extraction and elution of estradiol from an aptamer, is not the full story. As reported in Section 2.4.9, additional steps are required prior to analysis of the estradiol by GC/MS. These included liquid–liquid extraction of the estradiol from the Elution Buffer using hexane, followed by evaporative drying and derivatisation.

The limit of quantification for the analysis of derivatised estradiol by GC/MS was shown in Section 5.2.1 to fall in the middle of the range of material that might be expected to be obtained from 1 mL of serum. The high salt content in the buffers used was also thought to have a negative influence on the derivatisation procedure. It was thus deemed necessary to explore LC/MS/MS as alternative analysis tool, as this would eliminate the derivatisation and drying procedure as well as having lower levels of quantitation than GC/MS.

5.2.3. Evaluating the LC/MS/MS response to different quantities of estradiol present in standard solutions

Prior to analysing the serial dilutions of estradiol, the mobile phase conditions were optimised. The most suitable mobile phase conditions, which gave the lowest baseline noise, most consistent retention times and peak shapes on a XBridge C18 (2.5 μ m, 2.1 x 50 mm) column, was achieved by using a 5 % to 90 % gradient of methanol in water, at a flow rate of 0.2 mL/min. The method started with the methanol concentration held at 5 % for 2 minutes. The methanol concentration was then ramped up to 90 % over 12 minutes. It was maintained at this concentration for 3 minutes before finally being returned to the initial conditions in preparation for the next sample.

As reported in Section 2.4.6, E2 was serially diluted in the mobile phase solution (5 % MeOH / 95 % deionised water) at concentrations of 100, 10, 1 and 0.1 ng/mL, respectively. Data acquisition was performed in negative ion mode and the peak area response was determined for 10 μ L injections of underivatised E2 standards, over the range of concentrations reported above, using the parameters summarised in Section 2.4.5.

As can be seen in Figure 5.7, the peak areas obtained for E2 showed good linearity for a four point calibration, with a squared correlation coefficient of 0.9999. 10 μ L injections of the above standard solutions equate to 1000, 100, 10 and 1 pg being injected in to the mass spectrometer. The signal to noise determined at 1 pg was 14, indicating that a 1 pg injection is at the limit of quantitation, under the conditions used.

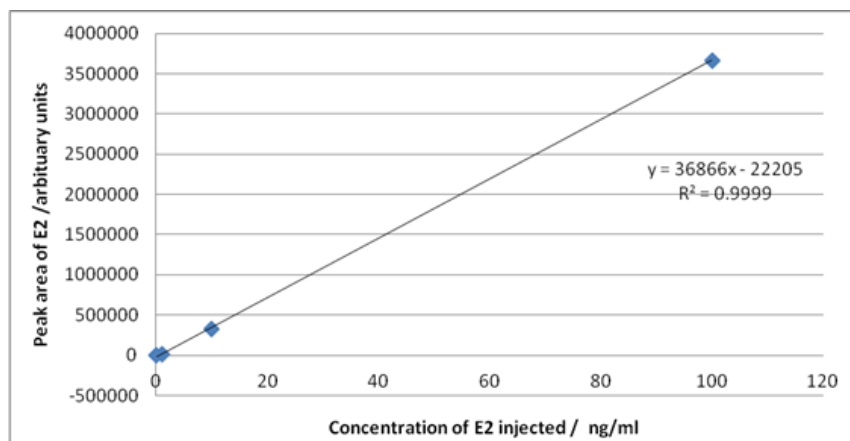


Figure 5.7. Showing the peak areas measured for 10 μ L injections of estradiol by LC/MS/MS over a concentration range of 100 ng/mL - 0.1 ng/mL

5.2.4. Optimisation of the solvent for extraction from the Elution Buffer prior to LC/MS/MS analysis

Prior to utilising the LC/MS/MS to evaluate the performance of the aptamer coated magnetic beads, the optimum solvent for the extraction of estradiol from the SDS Elution

Buffer was determined. Hexane and tert-butyl ether (TBE) were both evaluated as described in Section 2.4.12 and the chromatograms obtained are provided in Figure 5.8.

In brief, estradiol was spiked at the 1 $\mu\text{g/mL}$ level in 100 μL of the Elution Buffer. This solution was then diluted to 1 mL, with de-ionised water, before being extracted with the same volume of solvent (hexane or TBE). Once the extraction was complete the solvent was dried and reconstituted in 100 μL of the mobile phase of the LC/MS/MS (5 % MeOH, 95 % deionised water). 10 μL of the final solution was then injected, potentially equating to a 10 ng injection into the mass spectrometer.

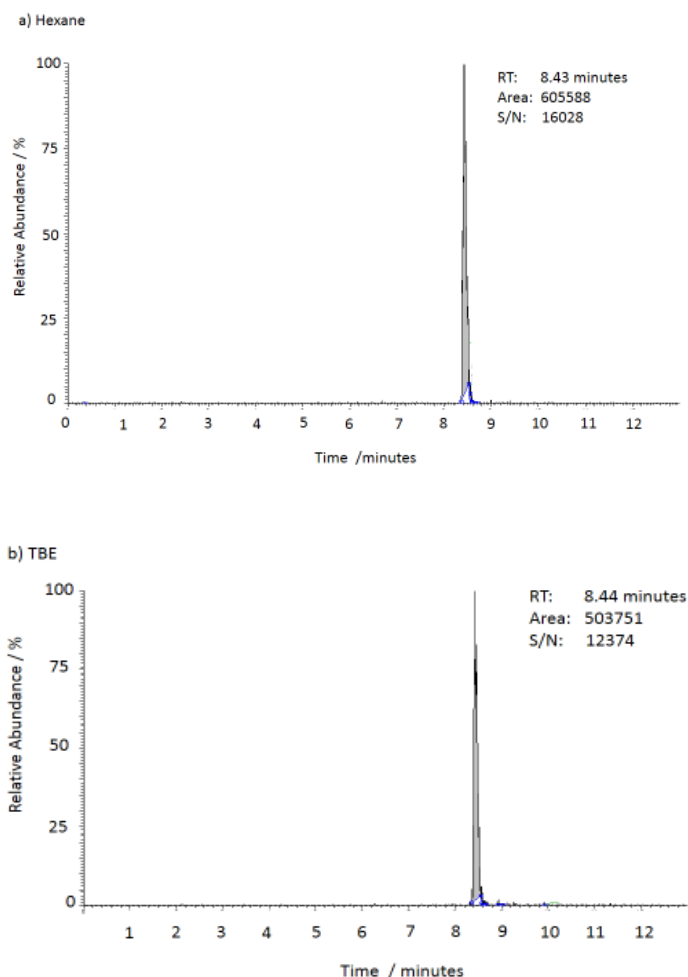


Figure 5.8. Example chromatograms obtained for estradiol following its extraction from the Elution Buffer in a) hexane and b) tert-butyl ether (TBE). Estradiol elutes at a) 8.43 and b) 8.44 minutes, respectively.

The analyses were repeated in triplicate for both solvents. The peak areas obtained for each extractions are shown in Figure 5.9.

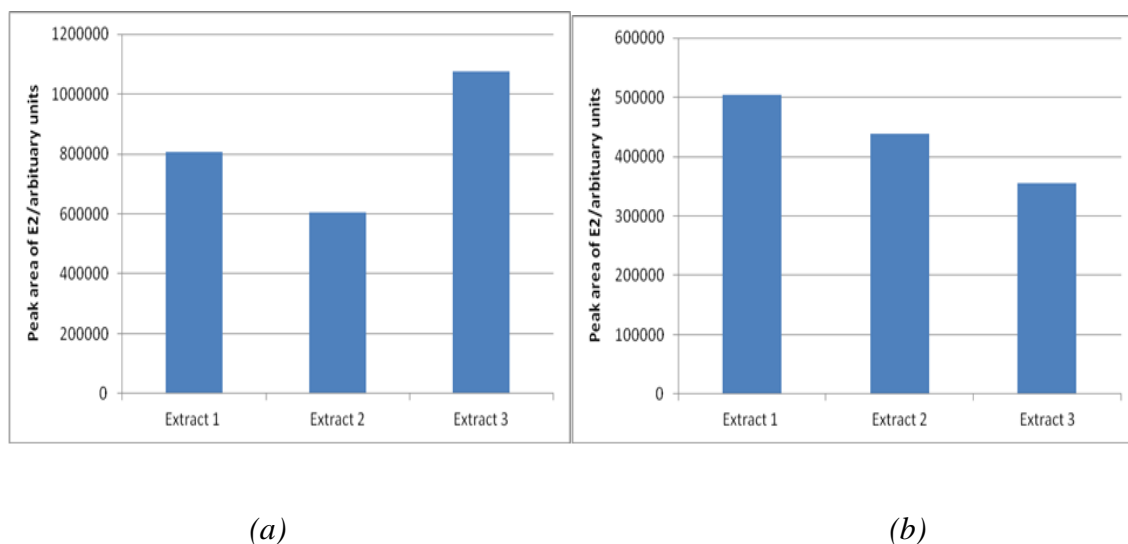


Figure 5.9. Peak areas obtained by LC/MS/MS for triplicate extractions of estradiol in: a) hexane and b) TBE.

As can be seen in Figure 5.9, when 100 μ L of the Elution Buffer is spiked at 1 μ g/mL, the average peak area obtained from extraction with hexane (828,353 \pm 235,058) is almost the double of that obtained when extraction is performed in to tertiary-butyl ether (432,192 \pm 74,689). Despite the greater repeatability of TBE, the higher recovery of hexane meant that the latter was used in the remainder of the study.

5.2.5. Evaluation of the aptamer extraction process using LC/MS/MS

The efficacy of the aptamer binding processes, as initially investigated by the GC/MS, was repeated using the LC/MS/MS. A number of different solutions were prepared for the evaluation and the peak areas were determined for the following solutions:

- i) 100 μ L of LC/MS/MS mobile phase (5% MeOH in deionized water) was spiked with estradiol at 1 ng/mL,

- ii) Hormone Binding Buffer was spiked with estradiol at 1 ng/mL,
- iii) Supernatant of the Hormone Binding Buffer, spiked with estradiol at 1 ng/mL,
following vortexing with aptamer coated magnetic beads for 1 hour,
- iv) Supernatant of an additional washing step using 100 μ L of the Hormone Binding Buffer, following vortexing for 2 minutes
- v) Supernatant of the Elution Buffer.

In contrast to the method illustrated in Figure 2.1, an additional wash step was introduced prior to the addition of the Elution Buffer. This step used 100 μ L of the Hormone Binding Buffer to evaluate if there was any non-specific binding of estradiol on the aptamers.

Apart from the initial mobile phase solution, the above solutions were prepared for analysis by LC/MS/MS, as described in Section 2.3.10. The supernatants were removed from the aptamer vial and diluted to 1 mL with dionised water. The estradiol was then extracted in to 1 mL of hexane, the hexane was dried and reconstituted in to 100 μ L of the mobile phase for analysis. Table 5.1 summarises the peak areas obtained for estradiol from each of the above solutions. It also relates the areas obtained for each of the solutions to the peak area obtained from spiking 100 μ L of the mobile phase with 1 ng/mL of estradiol.

Table 5.1 A summary of the peak areas measured for estradiol in each of the solutions and supernatants and their percentage recovery relative to a sample of the mobile phase spiked with 1 ng/mL of estradiol.

Spiked Solutions / Supernatants	Peak area of estradiol (arbitrary units)	Percent recovery of estradiol relative to mobile phase (%)
Mobile Phase	27770	-
Hormone Binding Buffer (before beads)	7338	26.4
Hormone Binding Buffer (after beads)	129	0.46
Washing Step	1088	3.9
Elution Buffer	8859	31.9

The results in Table 5.1 raise a number of questions. The peak area measured for the Hormone Binding Buffer, without exposure to the aptamer, is only 26.4 % of that determined from the direct spiking of the mobile phase with estradiol. This would suggest that either:

- i) the extraction of estradiol from the buffer in to the mobile phase is not complete, or
- ii) the process results in the transfer of species which can then influence the measurement of the target species in the LC/MS/MS.

A reduction in the anticipated peak area is not limited to the Hormone Binding Buffer, it is also found for the SDS Elution Buffer. This interesting finding will be returned to once the primary question for the experiment has been explored. The influence of the aptamer coated magnetic bead on the estradiol concentration will now need to be evaluated by considering the peak areas for each stage relative to the peak area determined for the spiked Hormone Binding Buffer that wasn't exposed to the aptamers. The relative percentage recovery determined for each step is summarised in Table 5.2.

Table 5.2 A summary of the peak areas measured for estradiol in each of the supernatants and their percentage recovery relative to the Hormone Binding Buffer.

Spiked Solutions / Supernatants	Peak area of estradiol (arbitrary units)	Percent recovery of estradiol relative to Hormone Binding Buffer (no beads) (%)
Hormone Binding Buffer (before beads)	7338	-
Hormone Binding Buffer (after beads)	129	1.8
Wash Step	1088	14.7
Elution Buffer	8859	120.7

The relative peak areas summarized in Table 5.2, provide an interesting insight in to the influence of the aptamer on the spiked solution of estradiol. The supernatant generated

following sixty minutes of vortexing of the spiked Hormone Binding Buffer with aptamer coated magnetic beads generated a signal that was less than 2% of that found in an equivalent volume of the spiked buffer. This result confirms the findings from the GC/MS study, though the lower detection limit of the LC/MS/MS has allowed the determination that the extraction is not totally complete. The signal to noise for the estradiol peak in this first supernatant is only 18, and so is approaching the limit of the level of quantitation.

The extra wash step was introduced as it was thought that it might be useful in the future to remove any matrix species that were non-selectively adsorbed during the first extraction. It was also introduced at this stage to evaluate whether any of the estradiol was not securely bound to the aptamer. Vortexing of the aptamer with 100 μ L of fresh Hormone Binding Buffer for 2 minutes resulted in the removal of approximately 15% of the estradiol. The loss of estradiol during this process will need to be further investigated and the reproducibility of this loss will need to be determined as it will influence the quantitative nature of the process. The loss of estradiol in this step will need to be considered against the benefits of the removal of matrix species and depending on the trade-off it may, or may not, be utilized.

Aptamers bind to their target due to shape-shape/hydrophobic/Van der Waals and ionic interactions.⁵ In some instances, as in this study, the binding can be very strong and is dependent on the buffer system used to select for the target. Aptamers are strongly influenced by the buffer system used and the binding will dictate the affinity for the target analyte. Each aptamer behaves uniquely taking on different structures and binding to their target by adopting various configurations. As in this study, these interactions can result in good affinity and specificity. Again this is largely due to shape-shape interactions *i.e.* surface complementarity and the structure the aptamer adopts is down to the buffer it is in -

salts such as NaCl, MgCl₂ and KCl, as used in the Hormone Binding Buffer, facilitate aptamers in adopting secondary structures that enhance the interactions with the target species.

The SDS Elution Buffer, which has a different composition to the Hormone Binding Buffer, provided a peak area for estradiol that was comparable to that found in the initial spiked buffer. Indeed the recovery was determined as being 121%. This slightly high percentage recovery may simply be a result of experimental error, but also it may have been a result of the influence that different buffers appear to have on the extraction of estradiol into hexane and subsequently the mobile phase introduced into the LC/MS/MS. The result obtained for the SDS supernatant would appear to be consistent with the earlier findings that indicated that there is significant loss in the estradiol signal from the introduction of the buffer solutions and the liquid-liquid extraction process.

As previously discussed, despite the high selectivity of LC/MS/MS, interferences from co-eluting endogenous compounds, or species introduced during the extraction process, can affect the quantitative analysis with electrospray ionization (ESI) as they can cause ion-suppression or enhancement.^{6,7} In this study, the latter appears to be more important; however if the method is transferred to clinical samples then the former will become more important. The response for an analyte in ESI-MS can be strongly influenced by the existence of other electrolytes in the solution that enters the electrospray and may provide a mechanism to explain the loss in the estradiol signal on moving from the spiked mobile phase to the buffer solutions.

In ESI, an analyte is already in the ionized form and transferred into the gas phase as a charged molecule, therefore the process that leads to the formation of gas phase ions can be

easily disrupted by the presence of matrix components. Charge can also be lost through neutralization or charge transfer reactions due to the presence of interfering compounds.⁶

Co-extracted matrix components may compete for the available charges surrounding the molecule and the entrance to the droplet surface between the analyte and the matrix components. The subsequent droplets formed require charged analyte trapped inside the droplets to escape the surface and enter into the MS orifice.

High analyte concentrations can also limit the charge available on the ESI droplets. Furthermore endogenous compounds with very high surface activities at high concentration can increase the viscosity and the surface tension of the droplets to change the efficiency of their formation and evaporation, which, in turn, affects the amount of charged ions in the gas phase that are separated and detected by the mass analyser.^{6,7}

The use of aptamers with magnetic beads is a comparatively new technique. The number of publications that involve aptamer and magnetic beads coupled to mass spectroscopy detection method are very few and far between. A review of the literature revealed very few publications that have dealt with the coupling of aptamers and mass spectroscopy, especially for small molecules detection.^{8,9,10,11} Most of the publications have developed bead-based methods for larger molecules such as proteins in peanuts and insulin.^{8,9}

The literature search revealed 925 publications on the application of aptamers with thrombin. The success of HD-1, the aptamer for thrombin, has been widely attributed to the short sequence that results in higher yield and lower cost of this aptamer. HD-1 was the first aptamer selected toward a protein target and is extensively used as a model for aptamer-based sensor approaches.

Perhaps the most comparable work to this study is that by Huy *et al.*¹² The authors used a Shimadzu HPLC with UV detection at 275 nm, for the detection of estradiol extracted and eluted using a specific aptamer (50-NH₂ GCTTCCAGCTTATTGAATT ACACGCAGAGGGTAGCGGCTCTGCGCATTCAATTGCTGCGCGCTGAAGCGCGG AAGC-30; random ssDNA: 50-NH₂-T₁₂TGGAGTAGATTGGCCAACCCTTTT-30). A wavelength of 275 nm was selected as it is a wavelength which is above the UV cut-off value for most commonly used solvent including methanol and acetonitrile.¹³ Furthermore, SDS and Buffer salts are compatible with HPLC UV detection and are used as ion pair reagents to improve the peak shape of basic compounds that interact with residual silanol groups in HPLC columns.

Another important aspect of Huy's work was that the samples analysed were spiked river waters and the wash step was comparatively mild, as it used MilliQ water. Furthermore, comparatively milder elution conditions of 40 mM Tris-HCl, 10 mM EDTA, 3.5 mM urea, and 0.02 % Tween20 at a pH 8.0 were used. However, this method provided low percentage recovery of between 28.1-20.4 % over the range of 5-50 µM.

The addition, extraction and efficient elution of estradiol from aptamers requires the application of harsh conditions including the use of urea (original Elution Buffer), chaotropic salts or detergents (SDS). As neither SDS nor the original Elution Buffer are suitable for LC/MS/MS analysis, the liquid-liquid extraction used in this study was a necessity, even though it introduces issues that will need to be characterised. As no HPLC-UV detector was available at the time of the study, it was not possible to apply this to our work.

Perhaps one major advantage of Huy's elution conditions is the potential to reuse the aptamers. SDS is particularly harsh and it is difficult to remove from the aptamers, a soapy residue was seen to remain on our beads. Therefore, in this context, despite the lower recovery, their work may be a better compromise as it enables the beads to be re-used.

Since this study was conducted, methods have been developed that use magnetic beads but with alternative agents to aptamers. Quotient Research published work describing the addition of monoclonal and polyclonal antibodies (raised against SXN101959) to tosyl-activated magnetic beads.¹⁴ The attachment was confirmed by tryptic digestion and monitoring antibody specific tryptic peptides by LC/MS-MS/MS.¹⁴ Similarly, attaching C18 moieties to magnetic beads would be useful. In such a case, optimised solvent conditions could then be used without any of the issues of denaturing the aptamer or the requirement for aptamer compatible methods.

In 2007, Kim *et al.*, had described a cost-effective, electrochemical detection method for estradiol using a DNA aptamer immobilized on to a gold electrode chip.¹⁵ Single-stranded DNA aptamers that specifically bound to estradiol were selected by the SELEX (Systematic Evolution of Ligands by EXponential enrichment) method and isolated for use. The isolated DNA aptamer was then immobilised on to the gold electrode, using the avidin–biotin interaction. Cyclic voltammetry (CV) and square wave voltammetry (SWV) were used to evaluate the level of binding of estradiol to the aptamer. Binding of estradiol with the aptamer caused the current to decrease and the magnitude of this drop was shown to be proportional to the level of binding.

The authors described the approach as cost-effective because it did not require expensive dyes and instruments. The authors also concluded that a streptavidin-modified gold surface

provided better surface coverage than using avidin–biotin interaction. A surface plasmon resonance (SPR) system was then used to confirm and optimize the conditions for the DNA-aptamer immobilization on the gold surface.

The SPR analysis was performed on the immobilized gold chips with various concentrations (0.1, 1 and 4 μM) of the aptamer. The SPR responses increased as the aptamer concentration was increased. No response was seen from the DNA aptamer-immobilized SPR chip when 1 μM of estradiol was added. The authors went on to conclude that mass-dependant detection methods such as SPR were not actually suitable for the detection and quantitation of small organic chemicals.

Other issues also arose in the study. In assessing the specificity of this biosensor system for estradiol, the authors chose to use 2-methoxynaphthalene and 1-aminoanthraquinone as negative controls, owing to their structural similarity to estradiol. However, given these compound are not steroids, more precise cross-reactivity studies with structurally similar steroid such as testosterone, should have been conducted.

Non-specific absorption was also highlighted as the cause for high background signal caused by the binding buffer on the gold electrode. The authors also suggest optimising the concentration of aptamers since a lower concentration of aptamer may not be enough to produce an efficient electrochemical signal. Conversely too high a concentration of aptamers may dampen the electrochemical signal.

Prior to both these studies, Kim *et al.* had reported on a new approach for analysing the hormonal activity of estrogens, by binding a yellow fluorescence, protein-fused human estrogen receptor on to glass slides.¹⁶ Avidin was covalently immobilized on an amine-modified glass slide. A biotinylated DNA probe was then immobilized through the avidin-

biotin binding interaction. The estrogens were incubated at 37°C for 30 minutes and the fluorescence was directly read with a micro-plate reader. The intensity was shown to be estrogen-dependent and enabled the identification and quantification of the hormonal activities of diethylstilbestrol, 17 β -estradiol, ethynylestradiol, 4-hydroxy tamoxifen and clomiphene. The method provided estrogen-dependent sensitivity $\sim 10^{-13}$ M for estradiol.

Xin *et al.*, have described the use of a magnetic particle-based chemiluminescence immunoassay (CLIA) for the detection of estradiol in human sera.¹⁷ The assay was designed to specifically recognise E2 in the presence of estrone (E1), estriol (E3), dihydrotestosterone (DHT), androstenedione, and testosterone.

Sodium trichloroacetate (Na-TCA) was specifically mentioned as a blocking agent for direct analysis of E2 in human serum, without extraction. The method had a detection limit of 2.51 pg/mL with a larger working range of 15–1000 pg/mL. High recoveries of 93.3, 106 and 101 % and low coefficients of variation (<15%) were also reported. This method was then applied to the determination of E2 in 105 human sera and compared with commercial radioimmunoassay (RIA) kit, with a correlative coefficient of 0.9892. the authors proposed that the method could be used as a diagnostic kit for clinical analysis of E2 in human serum.

A recently published study reported on the application of covalently immobilized recombinant human sex hormone binding globulin (rhSHBG) on paramagnetic beads for the screening and identification of androgenic and estrogenic designer steroids in dietary supplements.¹⁸

Testosterone and estradiol circulate in the bloodstream, bound mostly to the sex hormone binding globulin (SHBG) and to a lesser extent to serum albumin and corticosteroid-

binding globulin (CBG). Only 1-2% are unbound and are thus able to enter a cell and activate the relevant receptor. SHBG inhibits the function of these hormones, albeit providing a mode of transport due to the hydrophobic nature of steroids.

The binding affinity of SHBG is strongest for dihydrotestosterone (DHT) followed by testosterone, androstenediol and then estradiol.¹⁹ The binding affinity for other prohormones, studied in the previous chapters, can be significantly different, for example DHEA is weakly bound to SHBG, whilst androstenedione does not bind.²⁰

The assay was performed in a 96-well plate and combined with the fast LC-MS, 96 measurements could be performed within 4 h. The concentration-dependent inhibition of the label by steroids in buffer and dietary supplements was demonstrated.

Impressively, the designer steroid tetrahydrogestrinone was identified in a spiked supplement. The author claims that the steroid-binding assay can be used for high-throughput screening of androgens, estrogens, and gestagens in dietary supplements. One point of note was the lack of detection of androst-4-ene-3 β -17 β -diol in sample. This compound was not found by the targeted LC-MS/MS method, because the MRM transitions for this compound were not acquired.

5.3. Conclusions

The preliminary data from the pilot study reported in this chapter set out to evaluate the potential of aptamer-coated magnetized beads as a simple, high affinity, highly selective, high specificity, low cost method for the analysis of estradiol prior to its detection and quantification by mass spectrometry.

The study revealed the following:

- The peak areas determined by GC/MS for derivatised estradiol show a linear correlation with the concentration of estradiol standard introduced, though the limit of quantification is not sufficient to enable this method to be used for the range of concentrations found in clinical samples, in particular post-menopausal women.
- The peak areas determined by LC/MS/MS for underivatised estradiol show a linear correlation with the concentration of estradiol standard introduced, the limit of quantification is sufficient to enable this method to be used for the range of concentrations found in clinical samples.
- Based on the percentage recovery of estradiol, as determined by GC/MS, SDS was preferred over the original Elution Buffer solution.
- In contrast to HPLC UV, prior to analysis by mass spectrometry estradiol had to be removed from the buffer solutions through extraction with an organic solvent, this was then dried and reconstituted in to the starting mobile (LC/MS) or derivatising agent (GC/MS). This essential step added complexity and introduced issues, in terms of quantitation.
- Hexane was shown to result in higher recovery than tertiary-butyl ether (TBE), when extracting estradiol from the SDS Elution Buffer.
- The peak area measured for the Hormone Binding Buffer, without exposure to the aptamer, was only 26.4 % of that determined from the direct spiking of the mobile phase with estradiol. A similar reduction in the anticipated peak area was also found for the SDS Elution Buffer. This would suggest that either:

- the extraction of estradiol from the buffer in to the mobile phase is not complete,
or
- the process results in the transfer of species which can then influence the measurement of the target species in the LC/MS/MS.
- The supernatant generated following sixty minutes of vortexing of the spiked Hormone Binding Buffer (1 ng/mL) with aptamer coated magnetic beads generated a signal that was less than 2% of that found in an equivalent volume of the spiked buffer, indicating a high affinity of the aptamer for estradiol.
- Vortexing of the aptamer with 100 μ L of fresh Hormone Binding Buffer for 2 minutes resulted in the removal of approximately 15% of the estradiol. The benefits of adding a wash step in terms of cleaning up the sample of matrix species will need to be evaluated against the knowledge that estradiol can be lost through its implementation.
- The percentage recovery of estradiol in the Elution Buffer was determined as being 121%, relative to the peak area measured in the Hormone Binding Buffer.
- The use of a surfactant (SDS) as the Elution Buffer was not without its issues, with a visible residue being left behind on the magnetic beads, limiting its multiple re-use, thus increasing the cost.

The proposed strategy was twofold:

- i) to use aptamer coated magnetic beads as a vehicle to develop a simple technique that did not require skilled operatives and procedures that could be potentially be used for high throughput;
- ii) to use mass spectrometry as the detection device for its high specificity
- iii) a publication

In reality, coupling of the magnetic beads coated with aptamers and mass spectrometry necessitated additional steps that added complexity and were not without issue. The buffer conditions required to bind and then elute estradiol from the aptamer were not consistent with mass spectrometry.

Since conducting the study, other methods have been developed, most notably the recently published study reporting on the application of covalently immobilized recombinant human sex hormone binding globulin (rhSHBG) on paramagnetic beads for the screening and identification of androgenic and estrogenic designer steroids in dietary supplements. This would suggest that the approach was valid; however the selection of the aptamer material could have been better.

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NB References marked with *** at the end are non-peer reviewed articles

6. Conclusions and Future Work

6.1. Introduction

Steroids are a large family of organic compounds that are highly important biological molecules that play significant parts in the functioning of many living organisms. Several hundred steroids are known to exhibit bioactivity within human physiology. Many are produced by the human body (endogenous) but many are exogenous, produced either synthetically or by other organisms. Anabolic steroids increase muscle and bone production and are highly regulated and for many years they have been one of the most detected drug classes in sports testing. Current regulations are prepared by the World Anti-Doping Agency (WADA) and their latest Prohibited List (2012) names 50+ compounds that must be closely monitored by sports testing laboratories.

There is considerable interest in the development of high throughput multi-component, chromatographic methods to screen for a large range of compounds. In an attempt to overcome the issues associated with traditional analysis techniques, this thesis has explored and evaluated two complimentary approaches (aptamers and MIPs) to the extraction of a range of steroids prior to their separation, detection and quantification by comprehensive gas chromatography/mass spectrometry or liquid chromatography/mass spectrometry. The steroids selected for the studies either had clinical relevance (estradiol) or are of interest in the context of sports testing applications (prohormones and designer steroids).

The individual chapters have presented detailed discussions and the conclusions for each of the studies conducted. The aim of this chapter is to:

- i. collate and summarise these findings,
- ii. provide feedback on how the methodology could be improved, and

- iii. provide suggestions on how the work might ultimately be applied to a wider range of projects, by building on the findings reported.

6.2. A summary of the conclusions from the studies

Taking a chronological perspective of the research activities reported in this thesis, then the data from the pilot study reported in Chapter 5 set out to evaluate whether aptamer-coated magnetized beads had the potential to provide a simple, high affinity, highly selective, high specificity, low cost method for the extraction of estradiol prior to its detection and quantification with mass spectrometry.

In reality, the coupling of the magnetic beads coated with aptamers and mass spectrometry necessitated the use of additional solvent extraction steps to the standard method applied previously. These additional steps added complexity and were not without issue. In particular the buffer conditions required to bind and then elute estradiol from the aptamer were not found to be consistent with analysis by mass spectrometry. The derivatisation of estradiol prior to analysis by GC/MS was negatively influenced by the salts present in the buffers and the limit of detection was not found to be adequate for the proposed clinical purpose. Even when estradiol was analysed underivatised by LC/MS/MS, the use of surfactants in the elution buffer created issues with the analysis and even left physical residues on the aptamer beads, preventing their re-use. Significant effort would have been required to develop the extra clean-up steps.

Having explored and discounted the use of aptamers, the attention of the research project turned to the development of a method for extracting unconjugated steroids from synthetic urine, using molecular imprinted polymers (MIPs) templated with testosterone.

Two variants of the MIPs were developed, using different monomers, and their performances evaluated against non-imprinted versions of the polymers. Uniquely, this work has combined the selectivity of molecular recognition polymers with the high resolving power of two-dimensional gas chromatography/time-of-flight mass spectrometry to enable the identification and quantitation of all the prohormones without the need for derivatisation.

Based on the initial findings in Chapter 3, it was possible to draw the following conclusions:

- The identification and quantification of the cohort of twelve prohormones by GC-MS, following derivatisation, is very difficult due to poor chromatographic resolution and the wide range of compounds generated during the derivatisation process. This confirmed the findings reported by others and this remained the case when the derivatised species were analysed using comprehensive chromatography.
- In contrast, the majority of the underivatised unconjugated prohormones in a mixed standard solution, were resolved using two-dimensional comprehensive chromatography. Whilst a few of the prohormones co-eluted in the total ion chromatogram; all of these species could be resolved and quantified using their individual pattern of fragmentation ions, which were diagnostic. All the prohormones were also significantly resolved from the matrix compounds and column bleed. The level of matrix compounds present was also much lower than those found in samples following derivatisation.

- The elution characteristics of the individual prohormones could be grouped based on which of the functional groups were present, especially those at the 3- and 17-positions.
- The peak areas measured for each unconjugated prohormone were linear over the range of 50 to 500 pg injected. This range is equivalent to a quantity of material that could be recovered from 1 mL of urine samples spiked at 1 to 10 ng/mL, using the extraction method developed in this thesis. This range all covers the minimum required performance limit (MRPL) levels of the two latest WADA Prohibitive Lists.
- It was also calculated that each of the twelve prohormones was likely to be detectable below ~30 pg on column, and quantitation should be possible below ~100 pg on column. All the prohormones should therefore be detectable well below the MRPL of 10 ng/mL, as defined by WADA in 2010 and reduced to 5 ng/mL in 2012. It is accepted that whilst the sensitivity of the method could be improved further it is likely that any analysis of urines samples will require the hydrolysis of conjugated steroids in real world samples.
- The imprinting of a polymer with testosterone, using methacrylic acid (MAA) as the monomer, did not result in a significant increase in the percentage recovery of the prohormones over the non-imprinted polymer; when used to extract the cohort of prohormones from 1 mL of synthetic urine spiked at 10 ng/mL.
- The imprinting of a polymer with testosterone, using 4-vinyl pyridine (4-VP) as the monomer, did result in a significant increase in the percentage recovery for all the prohormones when compared with the non-imprinted polymer. The difference was

interpreted as being as a result of the steroids having greater non-specific interactions with MAA and more specific interactions with the unsaturated groups of 4-VP.

When the same comprehensive chromatography method was then applied to designer steroid standards in Chapter 4, the following was concluded:

- Most of the cohort of six designer steroids elute in different regions to the prohormones. The exception is epitestosterone, the 17 alpha-epimer of testosterone.
 - a. Xtren and trenazone, having a similar estradiene configuration, elute in a similar area and are retained longer than the prohormones.
 - b. Epistane, with its bridging sulphur group, is retained on both columns for longer than either Xtren or trenazone. It also elutes later than gestrinone in the first dimension yet interestingly it elutes earlier on the second dimension column, which has a greater level of aromatic substituents.
 - c. Gestrinone and THG, being estratrienes have the longest elution times on the second dimension, taking longer to elute than the current modulation time allows. As a result of this ‘wrap around’, they are found in their own distinct region of the surface plot. Their greater retention is due to the fact that their three conjugated carbon-carbon double bonds and the ketone group at the 3-position have the greatest interaction with the aromatic substituents in the stationary phases.
- Retention times are consistent in the first dimension; however, due to modulation, small changes in the second dimension retention time are possible. The one

exception is gestrinone which elutes from the second dimension column at around the time the modulator switches, which can result in significant changes in its second dimension retention time. This does not tend to cause an issue as the full mass range of ions (m/z 50 to 521) are available and the compound can be identified by comparison of its fragmentation pattern with library values.

- The use of underivatised steroids produces high spectral similarity between the library fragmentation patterns and those found for the steroids in the samples, as it prevents ambiguous identification due to derivatisation issue. As expected, the similarity increases when the concentrations of the steroid increases, as the background noise becomes less significant.
- The areas measured for the individual quantitation ions for each of the prohormones and designer steroids has been shown to be correlated with the amount of steroid injected.
- Not all of the designer steroids were detectable above background noise, in all the standard solutions injected:
 - Xtren and trenazone were detectable at 25 pg.
 - Epistane was only detectable down to 100 pg.
 - THG was detectable at 50 pg but not at 25 pg
 - Gestrinone was only detectable down to 100 pg

When the same method was then applied to the extraction of the designer steroid standards from 1 mL of synthetic urine spiked at the 10 ng/mL level, the following was concluded:

- Whilst the average percentage recoveries for all the designer steroids, except for THG, were much lower than those obtained for the prohormones, the recoveries were consistent and reproducible.
- Epistane was not detected in any of the eluents from the MIPs cartridges, suggesting that it was not extracted from the urine. The most likely explanation is that this is due to it not sharing the same conformation as testosterone and therefore it does not bind efficiently in the templated cavities.
- Further studies will be necessary to determine batch-to-batch variability and to observe how the cartridges perform if they are re-used, and also to assess the potential influence in moving from synthetic to human urine samples and towards conjugated steroids. The availability of conjugated steroids was beyond the scope of this thesis.

It is clear that not all the designer steroids are compatible with the method developed and further work is required to fully characterise their performance for a range of compounds. The viability of the screening capability is determined by the response factor of the designer steroid in the MS and the recovery from the MIPs. Both epistane and gestrinone have very low recoveries from the MIPs and have the highest detection levels (100 pg on column for standards, equivalence of 2 ng/mL in urine). MIPs templated with testosterone are not applicable to epistane and it is highly unlikely to be applicable in sports testing for gestrinone. Xtren, trenazone and THG have acceptable recoveries and better response factors (equivalence of 0.5 ng/mL, 0.5 ng/mL and 1.0 ng/mL, respectively). When taking both in to consideration they should be able to be detected at the 5 ng/mL level in urine. However, as stated previously, this test is currently for unconjugated steroids and therefore

will require the urine sample to be processed, prior to extraction, especially with the lower MRPL levels now required by WADA since 2012.

Despite this draw back, the MIPs method removes matrix components and the power of comprehensive chromatography coupled to TOFMS, reduces the probability of co-elution of the target compounds with system blanks, matrix compounds, masking agents or thus, reducing the likelihood of obtaining false positive and false negative results. The resolving power of comprehensive gas chromatography coupled with the high acquisition rate of the TOFMS also makes it an ideal tool for the screening of unknown compounds. Capturing of the full mass spectrum also allows for retrospective analysis, should new compounds emerge and co-elute.

6.3. Suggested Improvements

Data management and quantitation of species using Leco Pegasus IV GC×GC/TOFMS have posed significant challenges in this study. Whilst qualitative production of surface plots that enable the location of specific peaks to be identified and visualised is very intuitive, quantitative data processing is particularly labour intensive. Better data handling functions within the ChromaTOF software, that accompanies the LECO Pegasus GC×GC/TOFMS systems, will be required if this method is ever to be used routinely. The data-processing features require better search capabilities and functionality for library matching to species that are unique to the analysis. For example, the manual input of diagnostic ion so that the relevant mass spectrum and peak position can be pin pointed within the two dimension chromatogram. The automated data-processing capabilities leaves much to yearn for and the similarity of the steroids has definitely challenged the software capabilities. Despite this, the use of the 'Region Classification' function was

much appreciated, as prior to data processing, a specific region of the surface plot (e.g. the prohormone or designer steroid region) can be assigned for data processing, greatly reducing the time taken to process a sample if this were not performed.

One way of improving the chromatographic resolution of the compounds would be to look at alternative modulators to the one used in this thesis. It has been shown, by colleagues, that the Zoex cryogenic modulator reduces tailing in the second dimension, improving peak shapes and the ability to resolve closely eluting compounds. Newer designs for the time of flight mass spectrometer are also becoming available, such as the BenchTOF-HD and BenchTOF-Select systems now sold by ALMSCO, a subsidiary of Markes International Ltd. The BenchTOF-Select allows the energy of the electrons in the ionisation source to be modified thus providing even greater selectivity.

Extending the modulation period would resolve the issues with gestrinone eluting at the point when the modulator switches from one process to another. Careful consideration will need to be given to what influence this has on all the steroids, as extending the modulation period will result in larger slices of the first dimension eluent being collected together.

An alternative approach to comprehensive chromatography would be to utilise high resolution/accurate mass MS systems that are becoming increasingly affordable. A high resolution/accurate mass system coupled to molecular imprinted polymers, could provide an opportunity to build on this study for steroid analysis. The specificity would result from accurate mass measurement of the molecular ions.

It should be noted that since conducting these experiments the WADA specifications for anabolic androgenic steroids have changed. It changed from a level of 10 ng/mL in 2010 to 5 ng/mL in 2012. It is therefore recommended that studies with prohormones and designer

steroids be replicated with the knowledge of this lower threshold value and that the method is evaluated by spiking urine samples obtained from humans.

6.4. Future Work

The results presented in this thesis and the publication to be found in Appendix A4 have illustrated that the coupling of MIPs (tailored to the target family of compounds of interest) with comprehensive gas chromatography/mass spectrometry provides a method that is capable of extracting, resolving and quantifying a cohort of unconjugated, underivatised steroids (prohormones and designer steroids) from synthetic urine, at concentrations that are appropriate for sports testing. The key to the successful application of this method will be its coupling with a method that hydrolyses conjugated steroids making them available for MIPs extraction and separation by GCxGC/TOFMS. It is understood that this work will form the basis of future application to WADA by the co-authors of the publication.

The ability to screen and resolve a wide range of very similar steroids has the potential to be applied in fields well beyond those explored during this thesis. For example, the results generated in this thesis have already formed the basis of:

- i. an application to BBSRC, to investigate the level of stress hormones released by fish. In partnership with the University of West of Scotland and the University of Bath the bid has been reviewed and was deemed “excellent and fundable” and the applicants await the decision of the funding panel. The bid title was “Trace detection of steroidal compounds in the aqueous environment using molecularly imprinted polymers and comprehensive GCxGC and LCxLC detection.”

- ii. discussions with Prof. Malcolm Mason (Cardiff University / Cancer Bank Wales) with the view to a joint application to the Cancer Research UK Biomarker Project Panel, in November, to evaluate the performance of the method developed in this thesis for screening urine samples for prostate cancer.

The projects named above are probably just the beginning of the journey for MIPs coupled with comprehensive chromatography. Indeed, feedback already received on the publication has indicated that there may be a significant range of applications in environmental monitoring. In addition, as intimated in the introduction to the thesis, the method developed could also be adapted to have applications in nutritional supplement testing.

Whilst the Leco Pegasus IV has provided the analytical basis of the studies reported in this thesis, its high cost and use of liquid nitrogen in the cryo-modulator will tend to preclude its adoption in mainstream laboratories. In contrast, add-on systems such as the Capillary Flow Technologies developed by Agilent or the Zoex cryo-modulator and associated software enable existing laboratory GC-MS systems to be effectively upgraded. These are not without limitations, but should be explored as they will provide niche solutions in specific application areas at a lower cost.

6.5. Summary

In summary, the thesis has described the development of a screening method capable of detecting multiple, unconjugated prohormones, steroids and designer steroids in urine that are structurally similar and commercially relevant at concentration levels that are applicable to sports testing. A molecularly imprinted solid phase extraction clean-up step was employed prior to injection on a GCxGC/MS. Significant improvements over commercial C-18 material were observed. The nature of this study also removes the need for sample

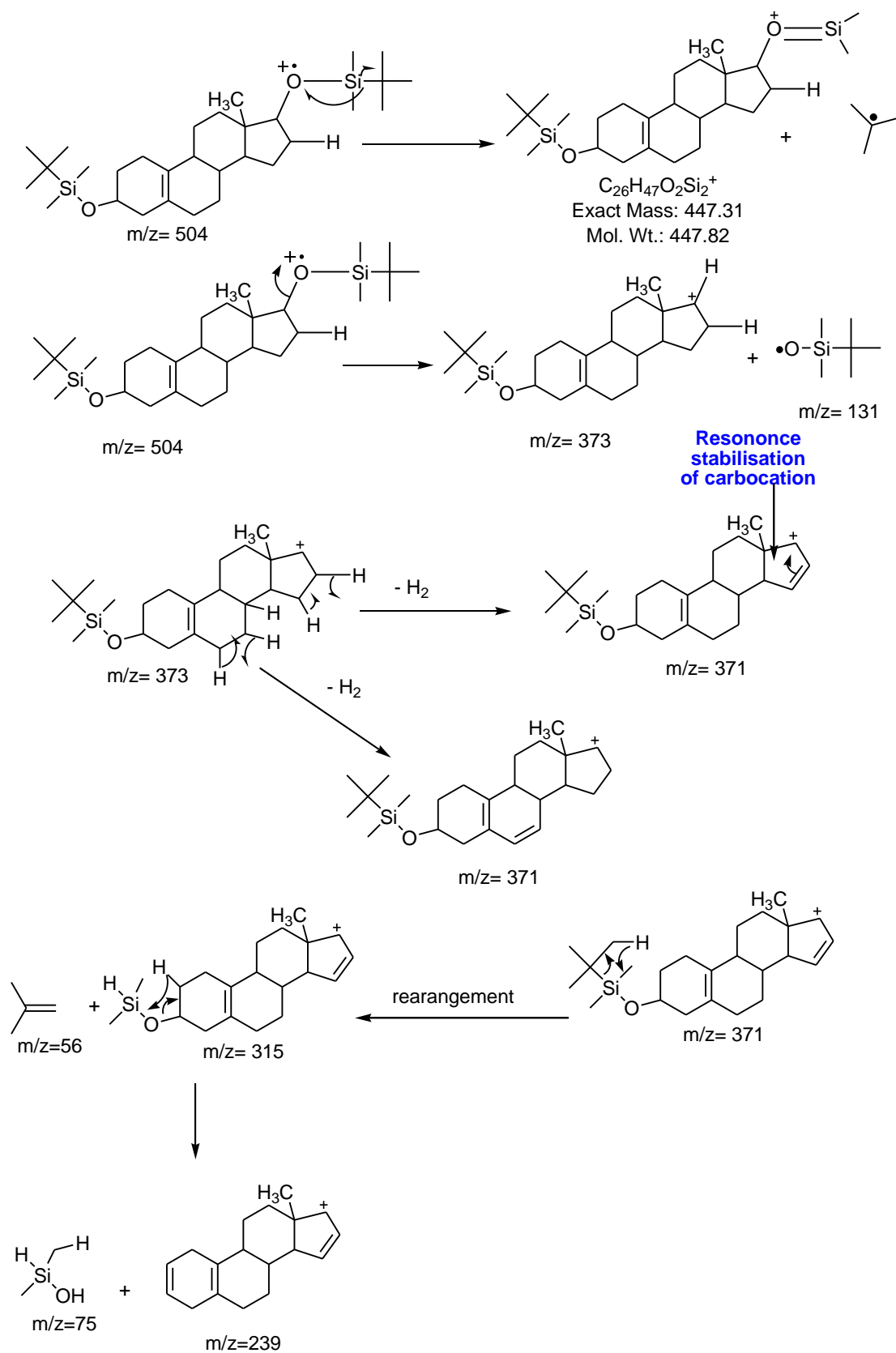
derivatisation, which speeds up the screening process. However, as the method is based on the extraction and separation of unconjugated steroids, for the technique to be adopted by the sports testing community and for it to meet the requirements of the WADA MRPL it will either require a suitable hydrolysis method to be developed or the sensitivity of the method will need to be further improved. Potential improvements have been suggested.

It is interesting to ponder whether THG would potentially have been detected sooner, if the technique developed here had been available along with a suitable hydrolysis method?

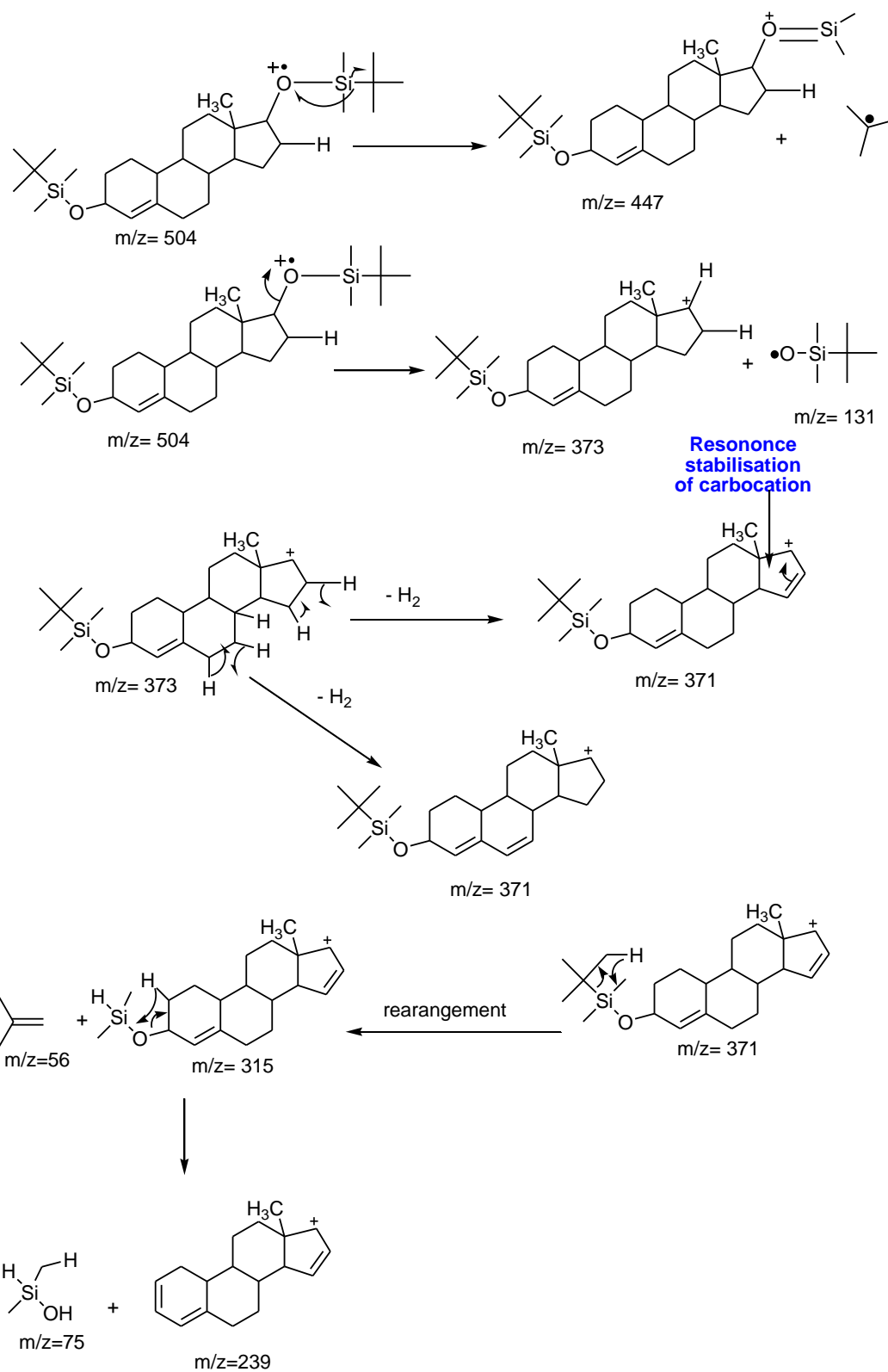
Appendix A1.1

Mechanisms proposed for the formation of the major fragment ions for the derivatised prohormones.

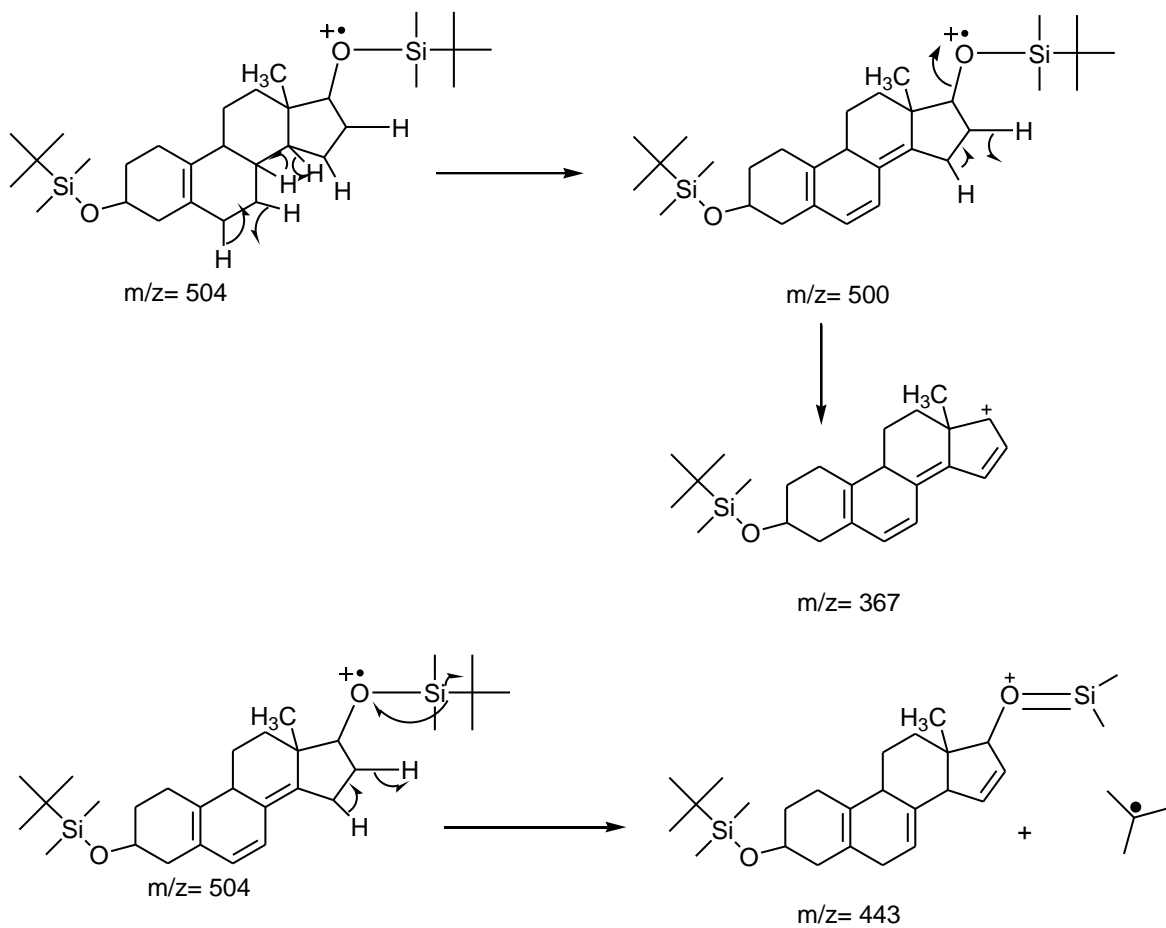
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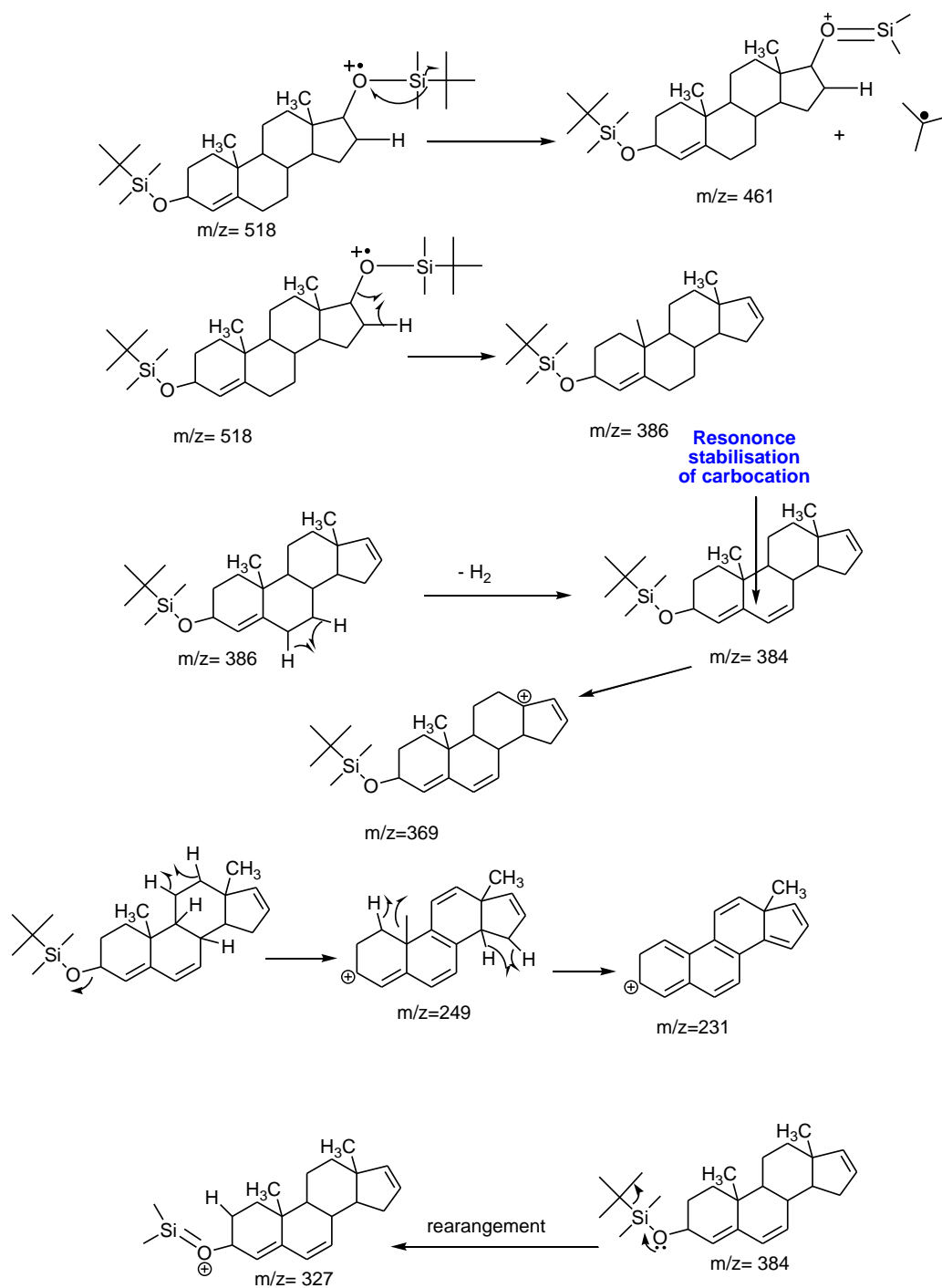
2. 4-estrene-3b,17b-diol, bis-(O-tert.-butyldimethylsilyl)-



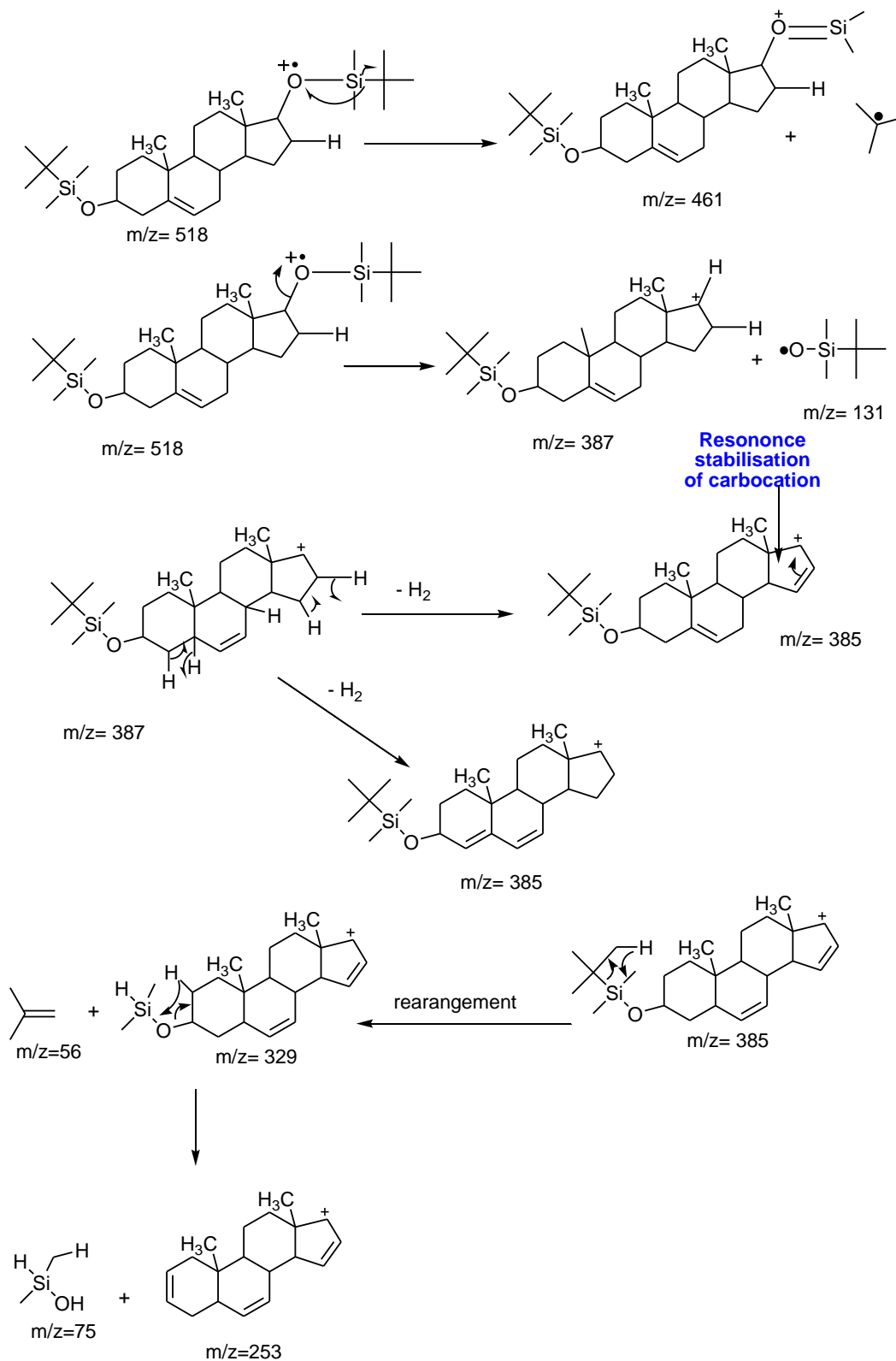
3. 5(10)-estrene-3b,17-dione,bis-(O-tert.-butyldimethylsilyl)-



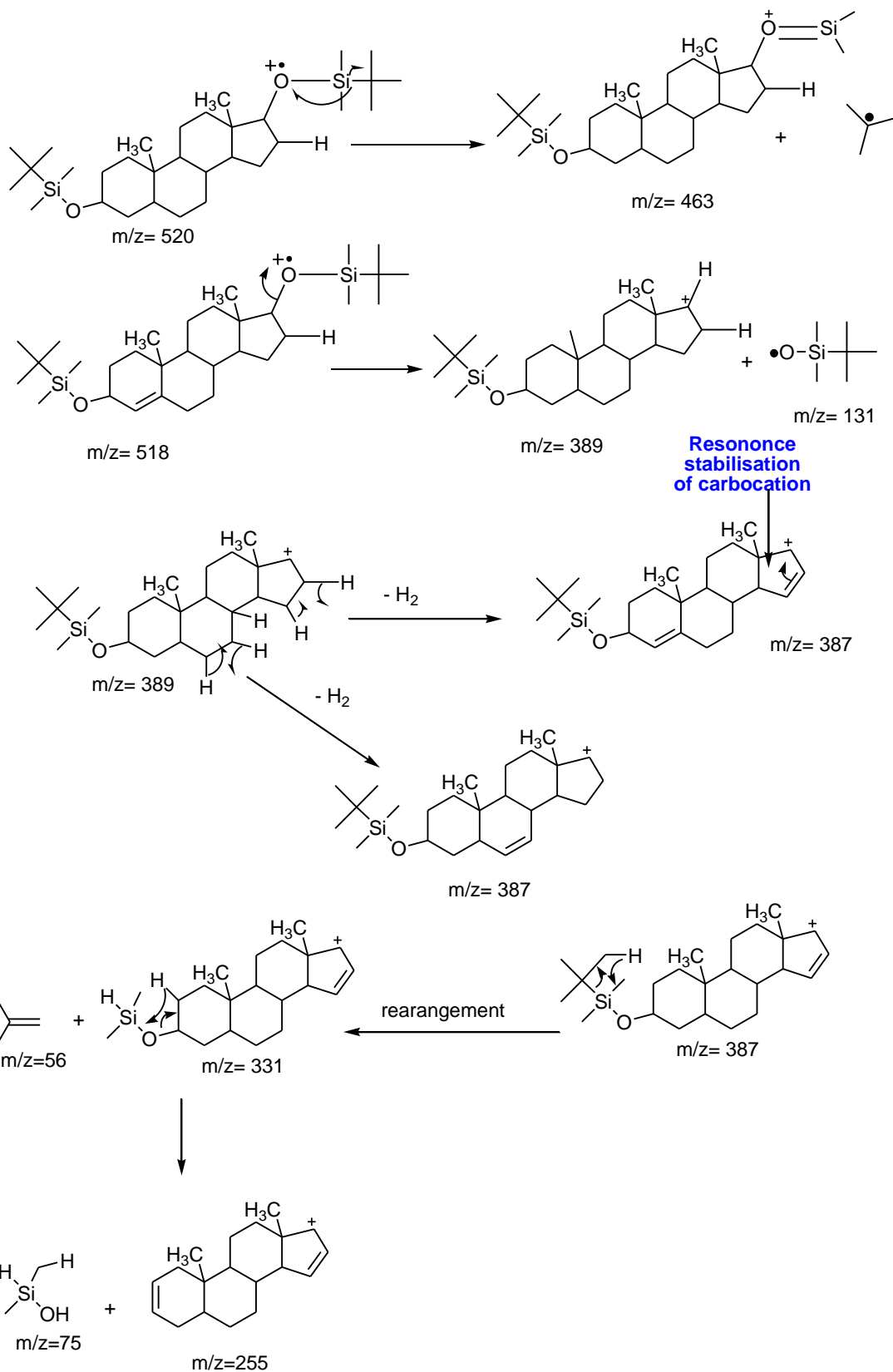
4. 4-androstene-3b,17b-diol, bis-(O-tert.-butyldimethylsilyl)-



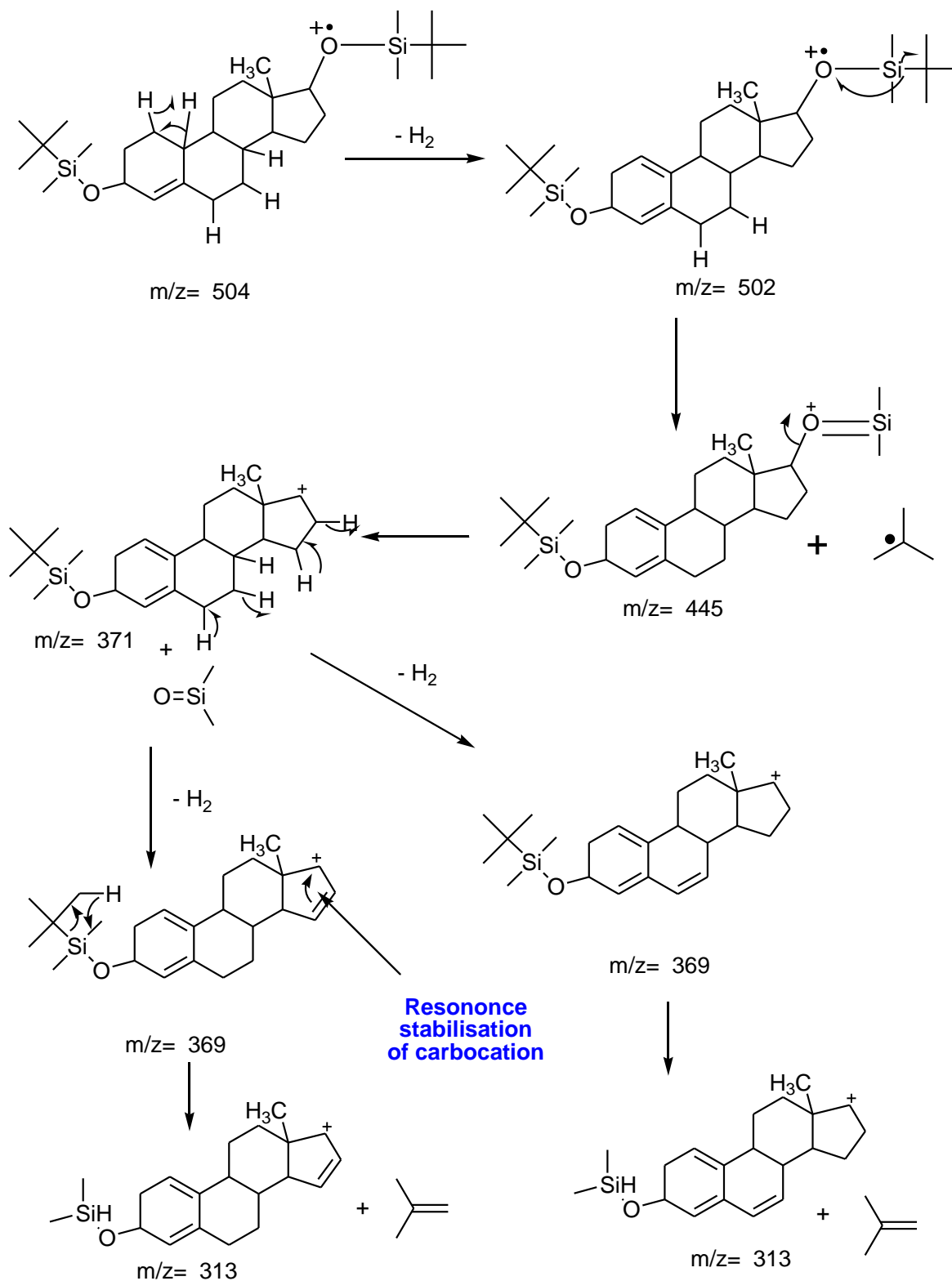
5. 5(6)-androstene-3b,17b-diol, bis-(O-tert.-butyldimethylsilyl)-



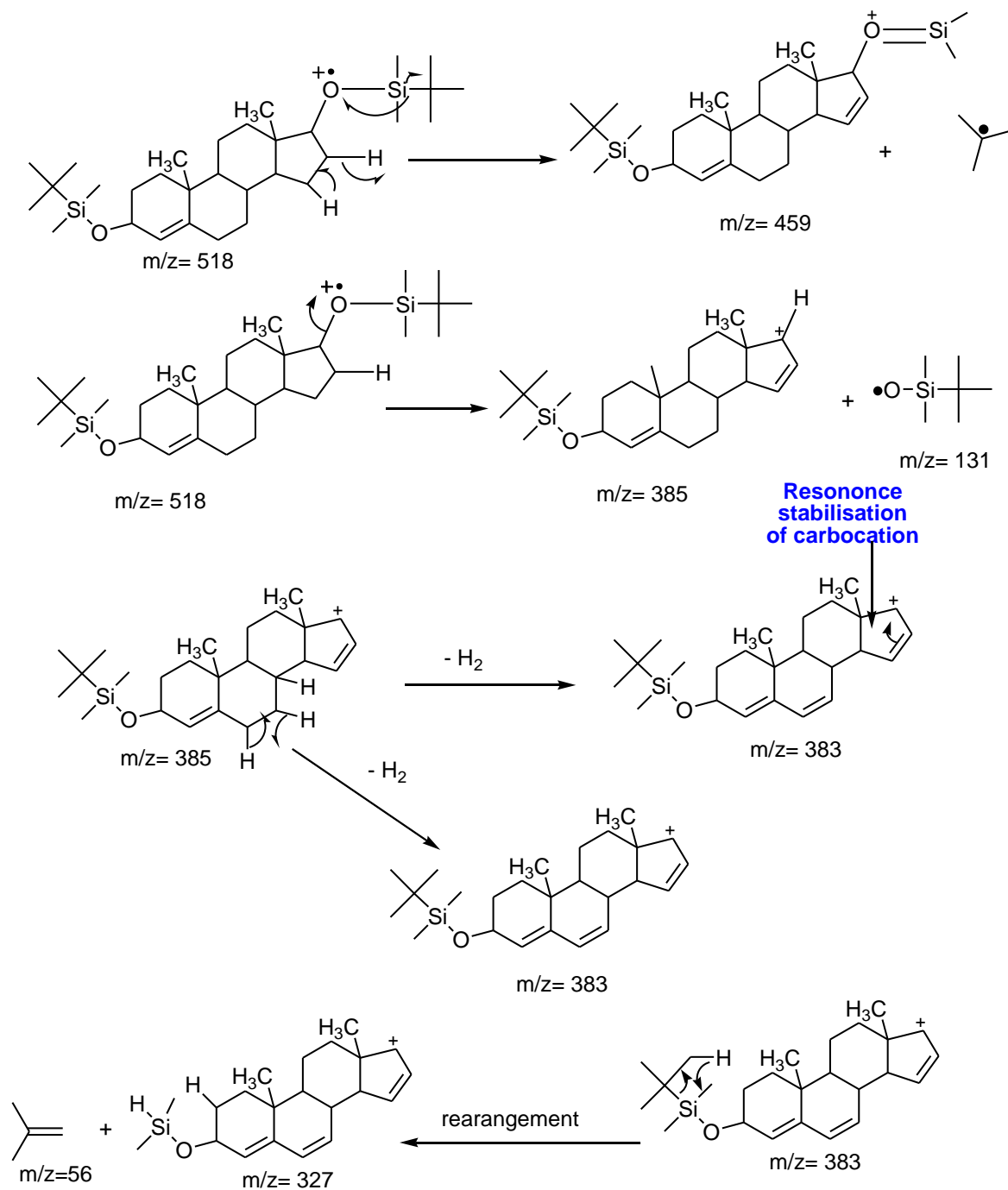
6. 5a-androstane-3b,17b-diol, bis-(O-tert.-butyldimethylsilyl)-



7. Nandrolone, bis-(O-tert.-butyldimethylsilyl)-



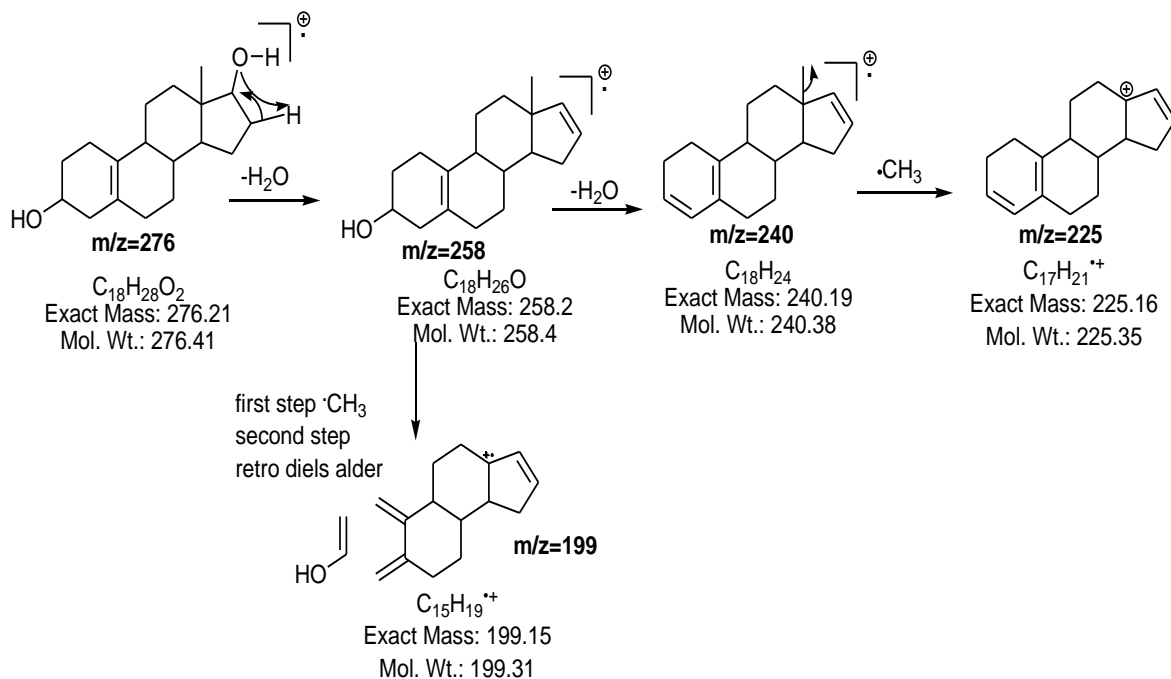
8. Testosterone, bis-(O-tert.-butyldimethylsilyl)-



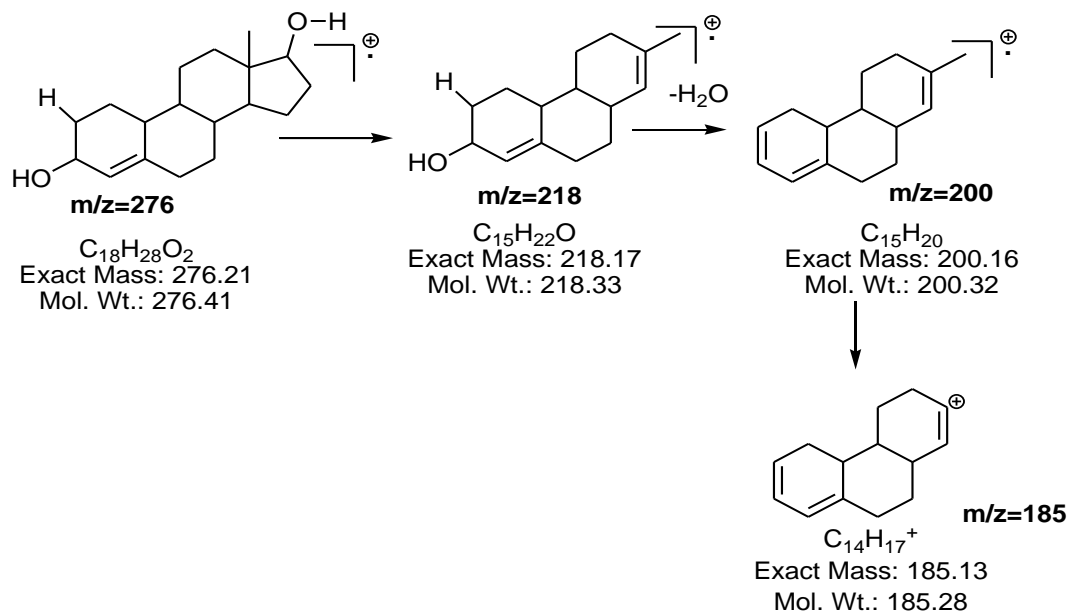
Appendix A1.2

Mechanisms proposed for the formation of the major fragment ions for underivatised prohormones

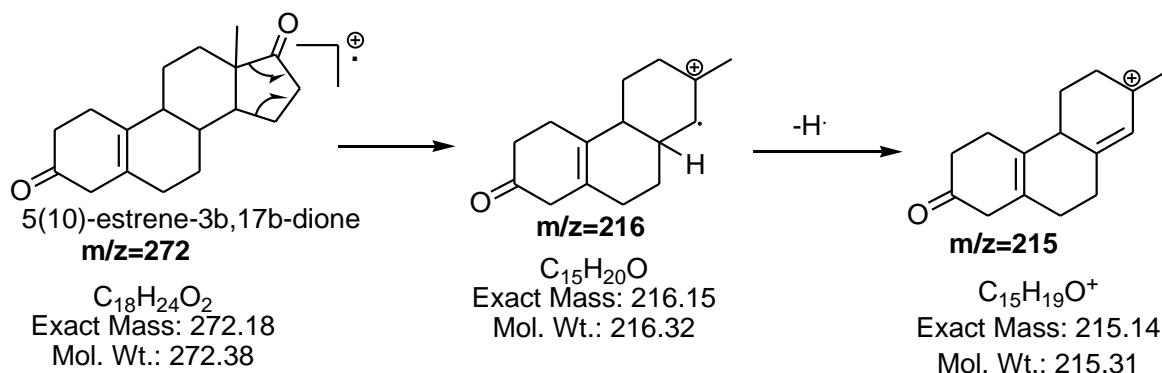
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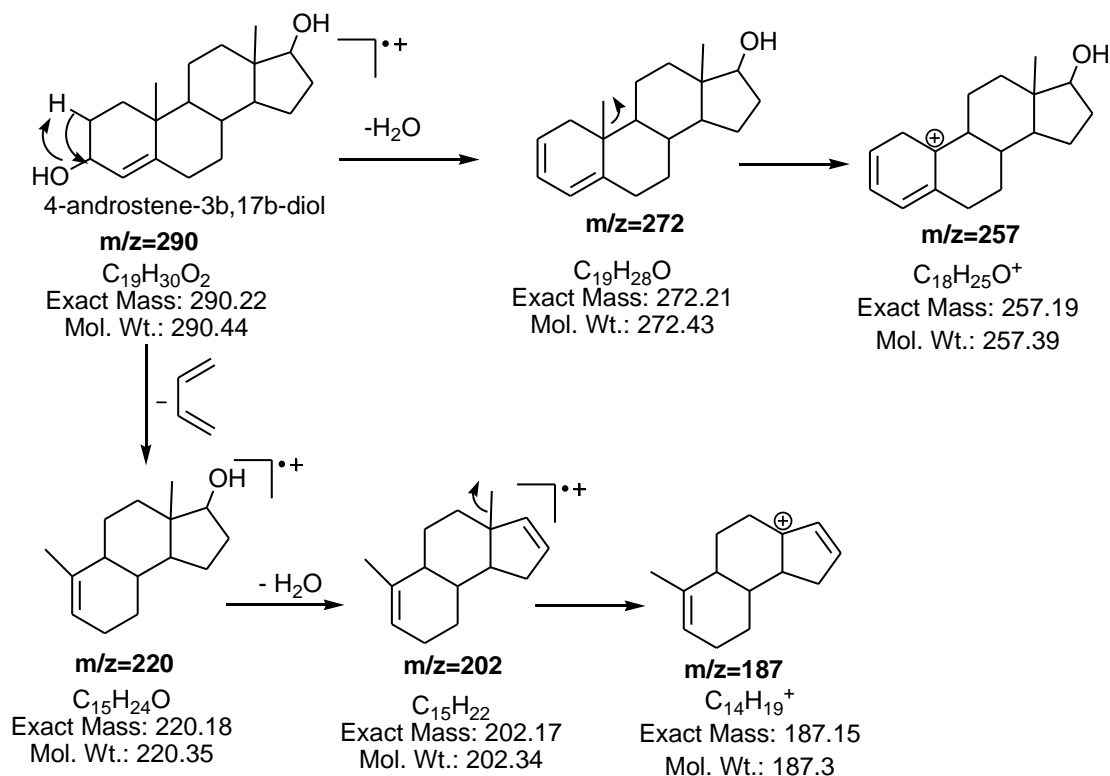
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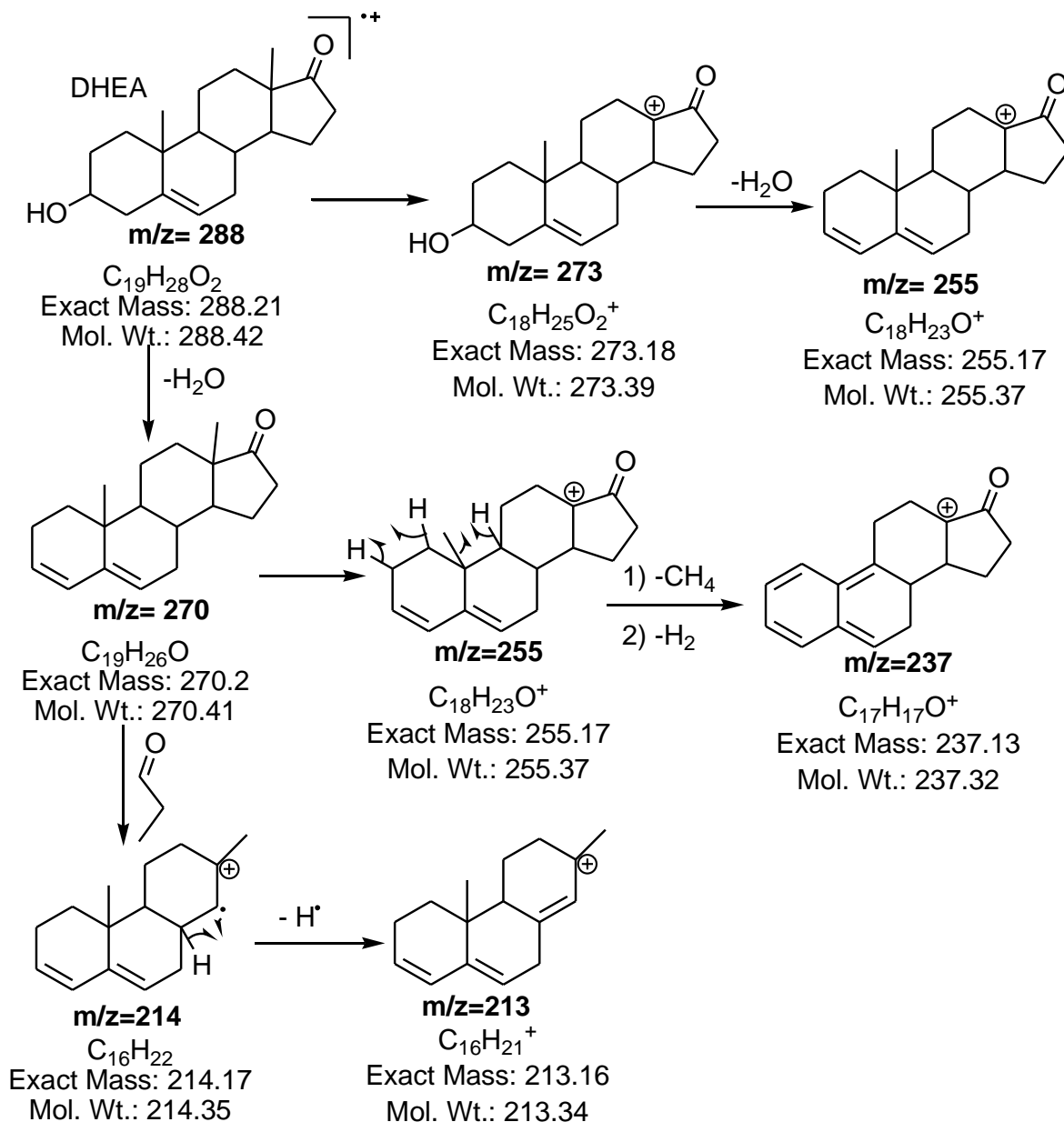
3. 5(10)-estrene-3b,17b-dione



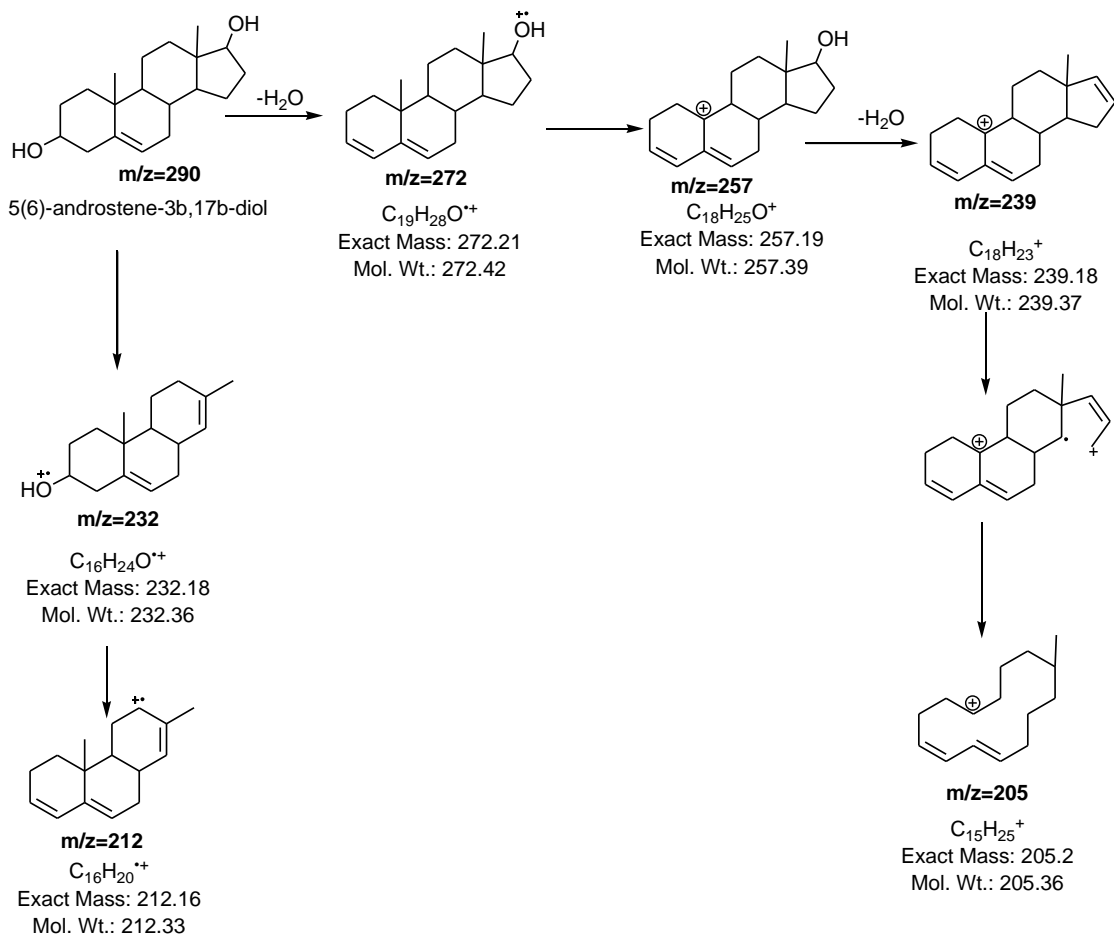
4. 4-androstene-3b,17b-diol



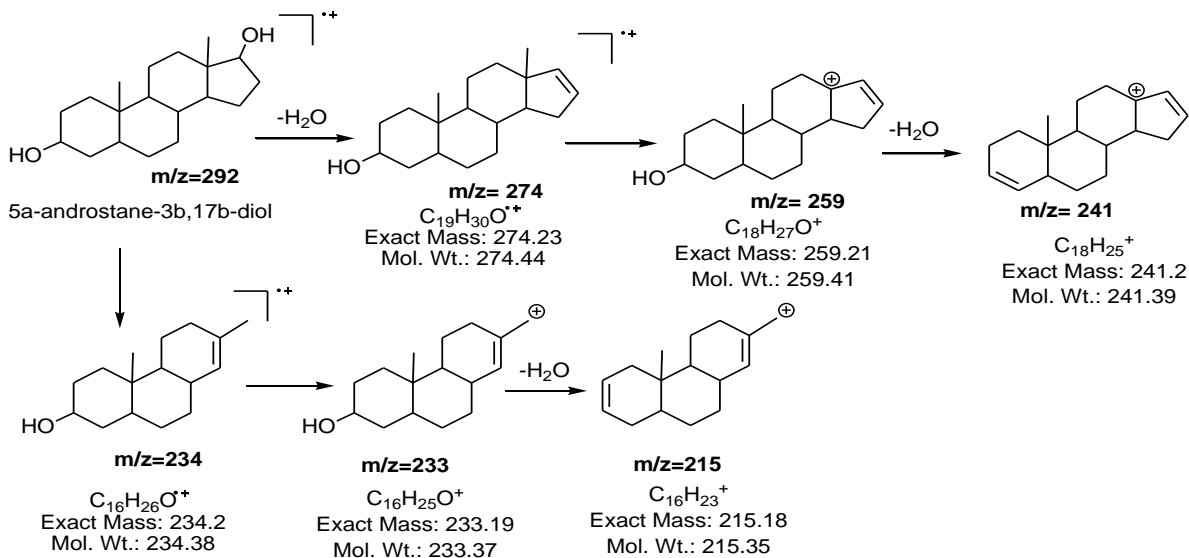
5. DHEA



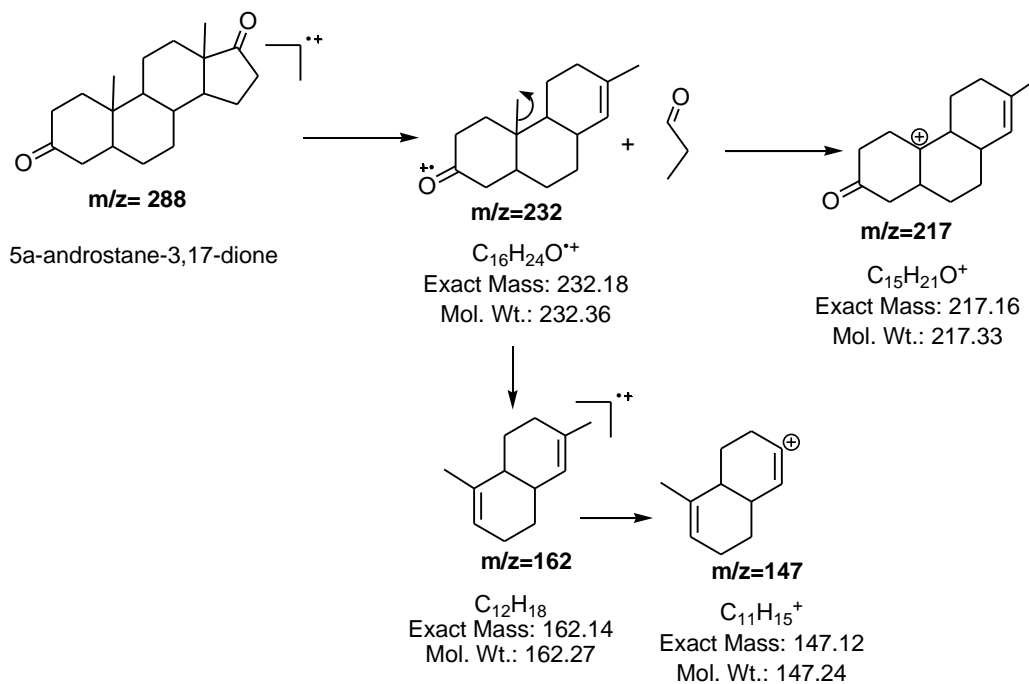
6. 5(6)-androstene-3b,17b-diol



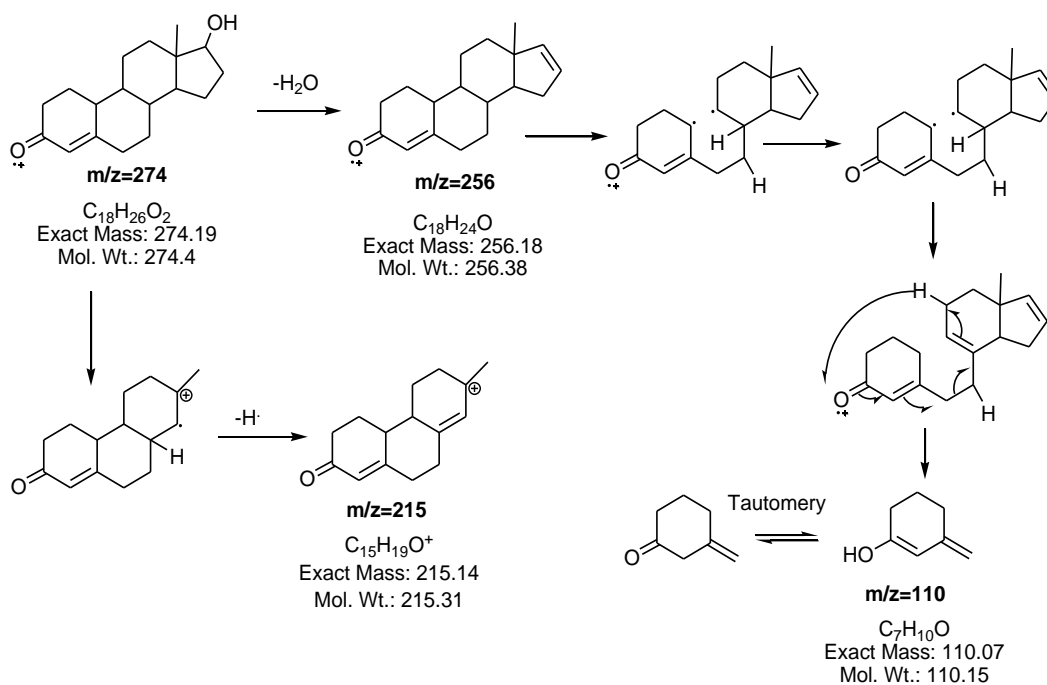
7. 5a-androstane-3b,17-diol



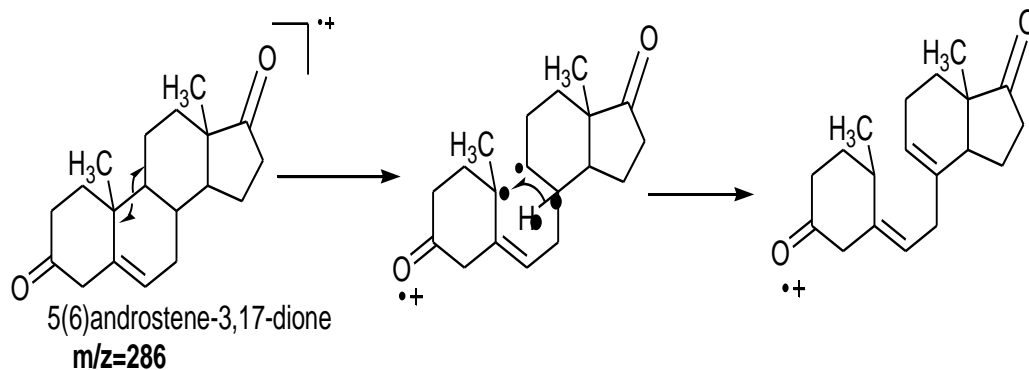
8. 5 α -androsterane-3,17-dione



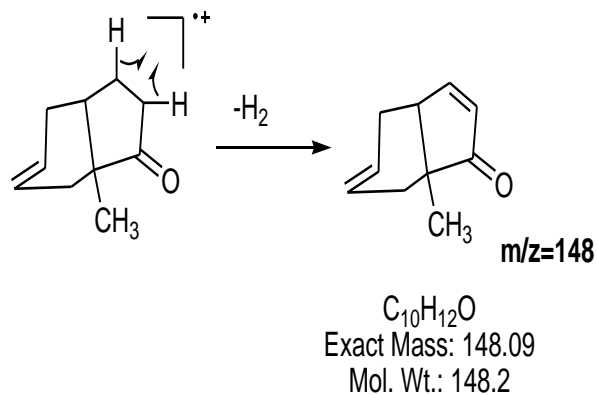
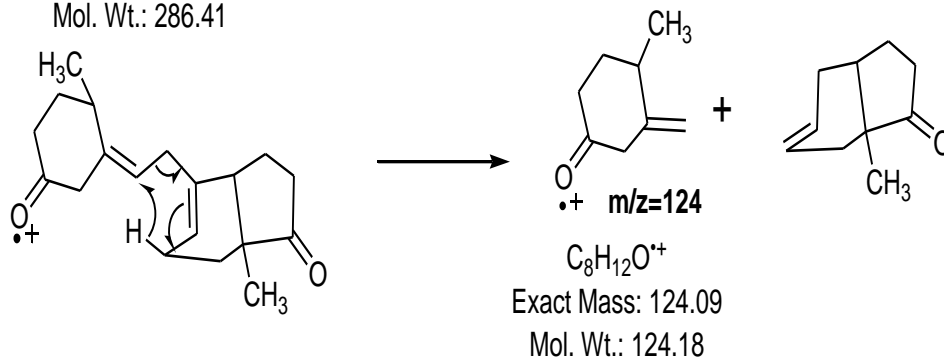
9. Nandrolone



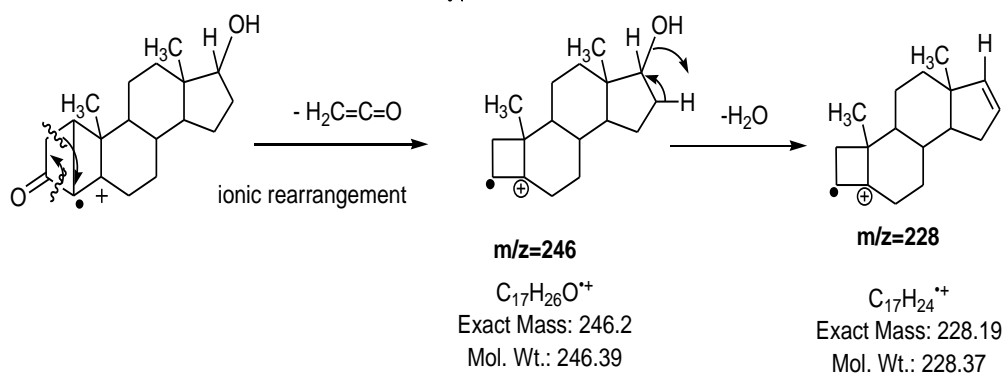
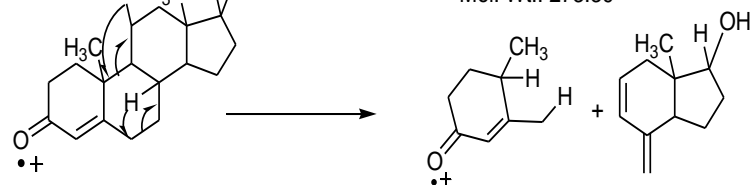
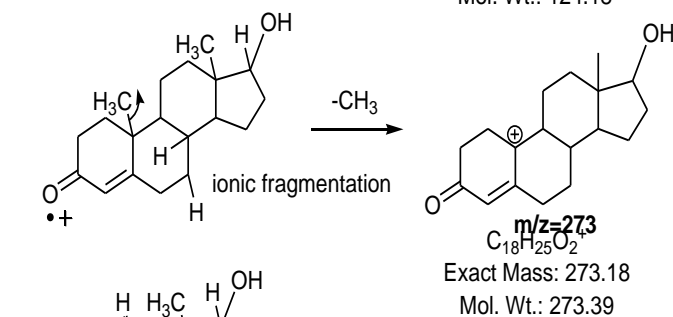
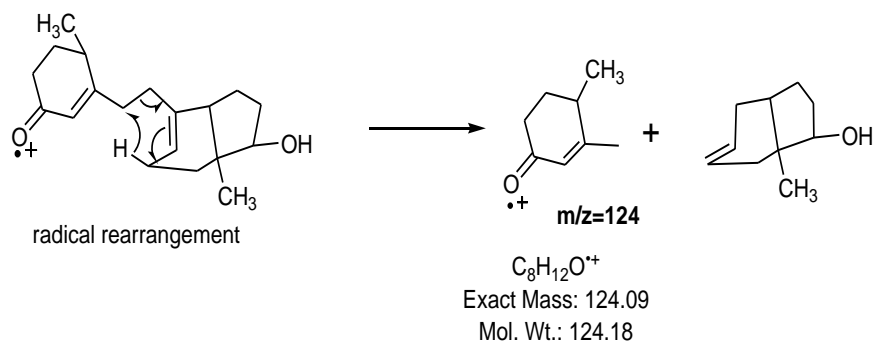
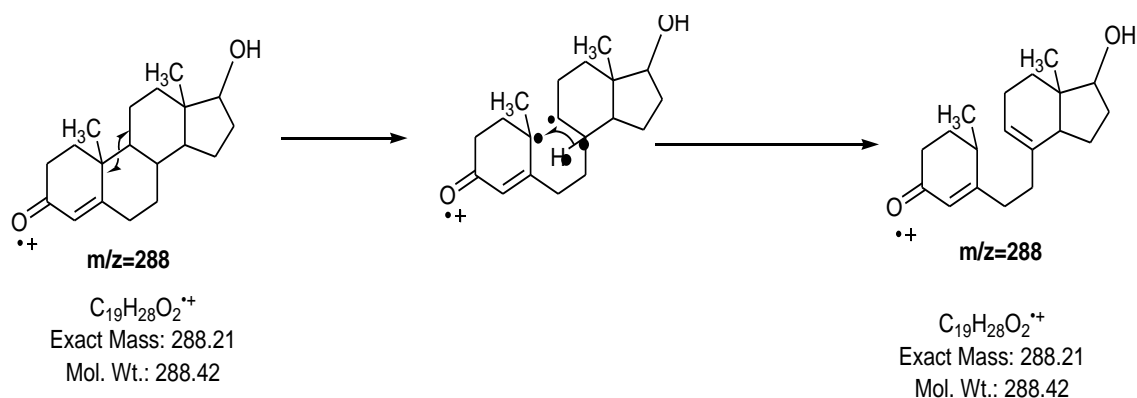
10. 5(6)-androstene-3,17-dione



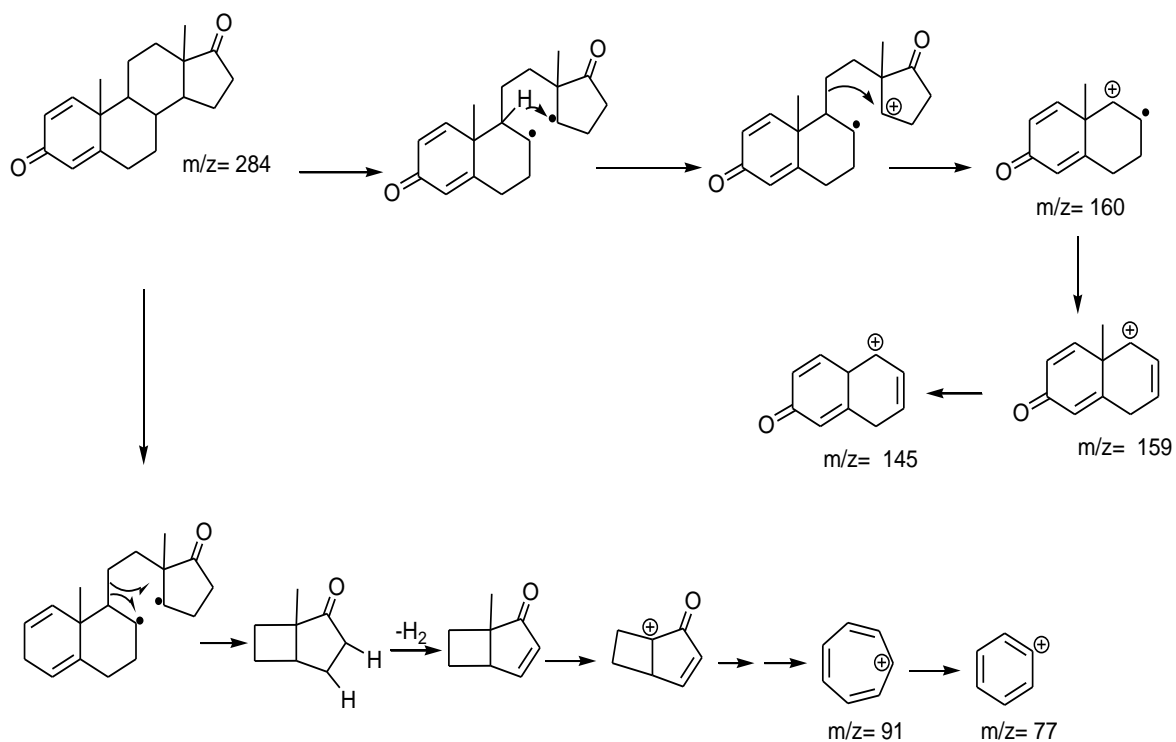
$C_{19}H_{26}O_2$
Exact Mass: 286.19
Mol. Wt.: 286.41



11. Testosterone



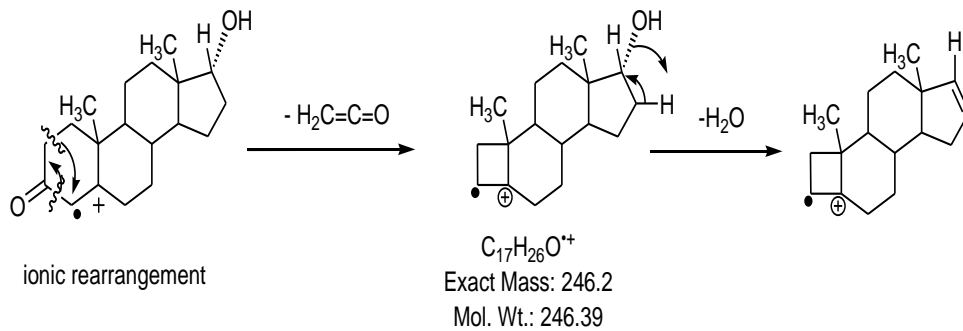
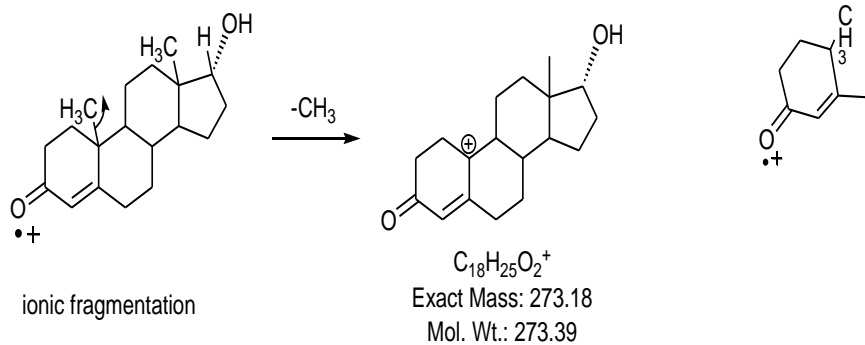
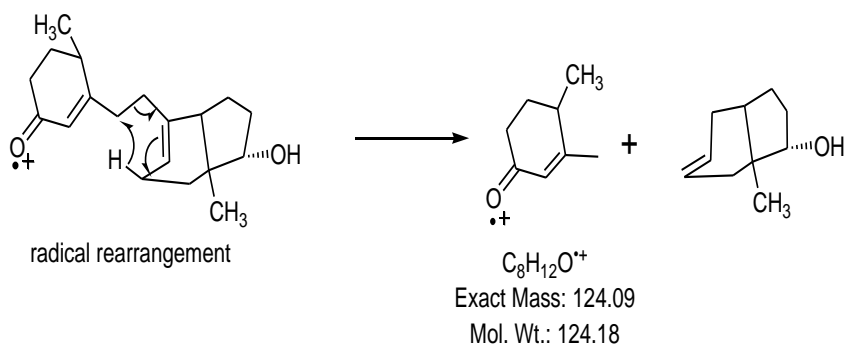
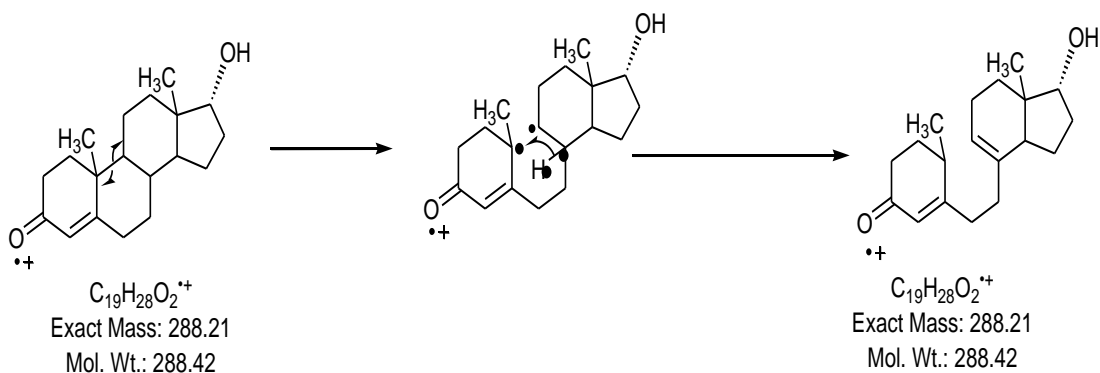
12. 1,4-androstadiene-3,17-dione



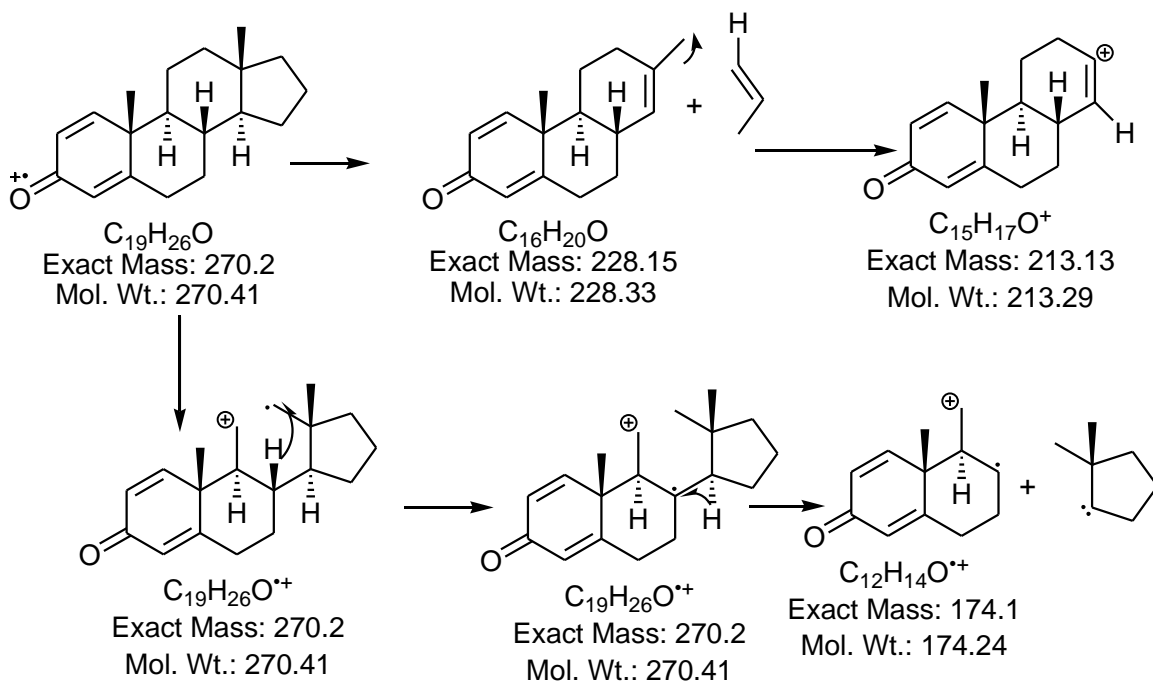
Appendix A1.3

Mechanisms proposed for the formation of the major fragment ions of the designer steroids

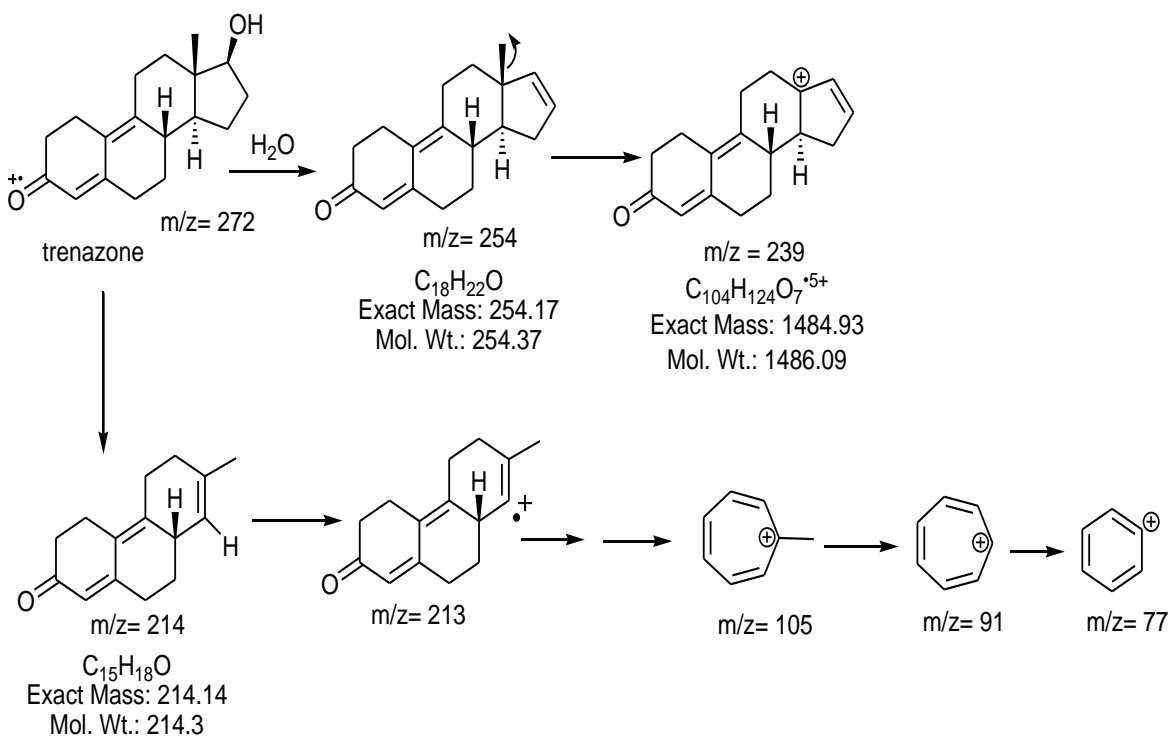
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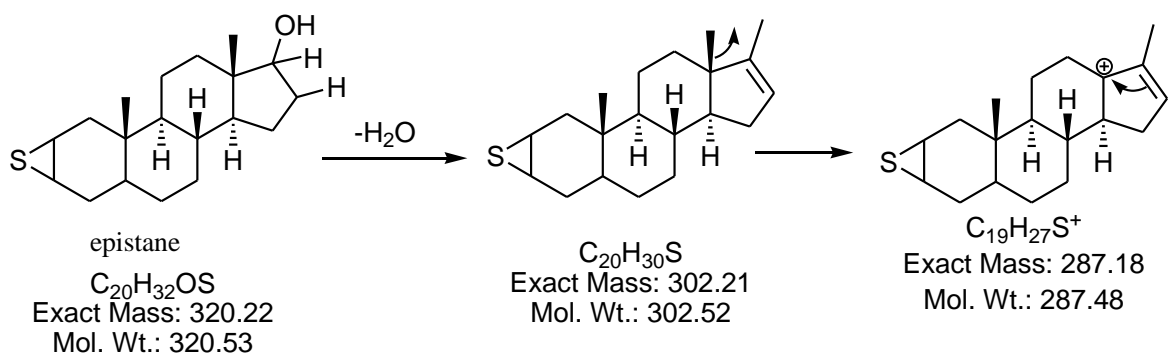
2. XTREN



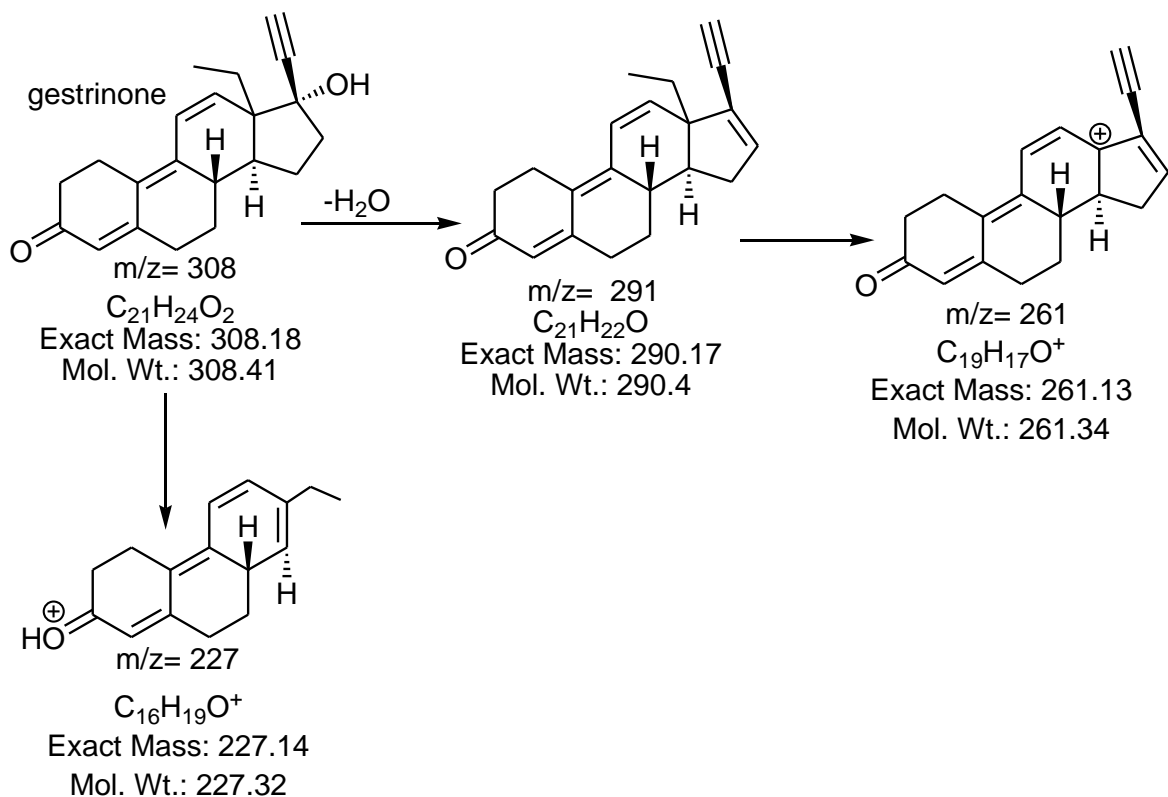
3. Trenazone



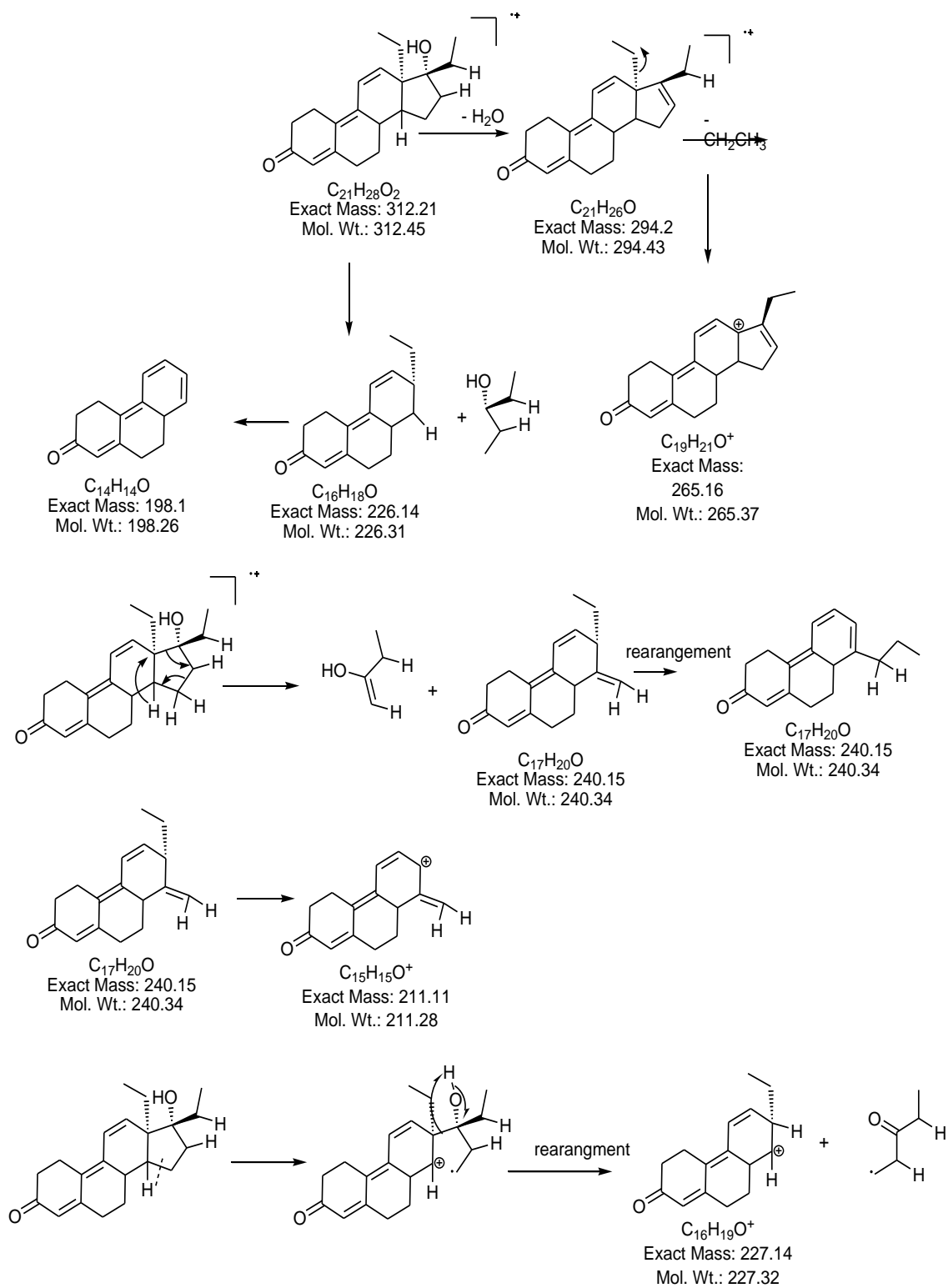
4. Epistane



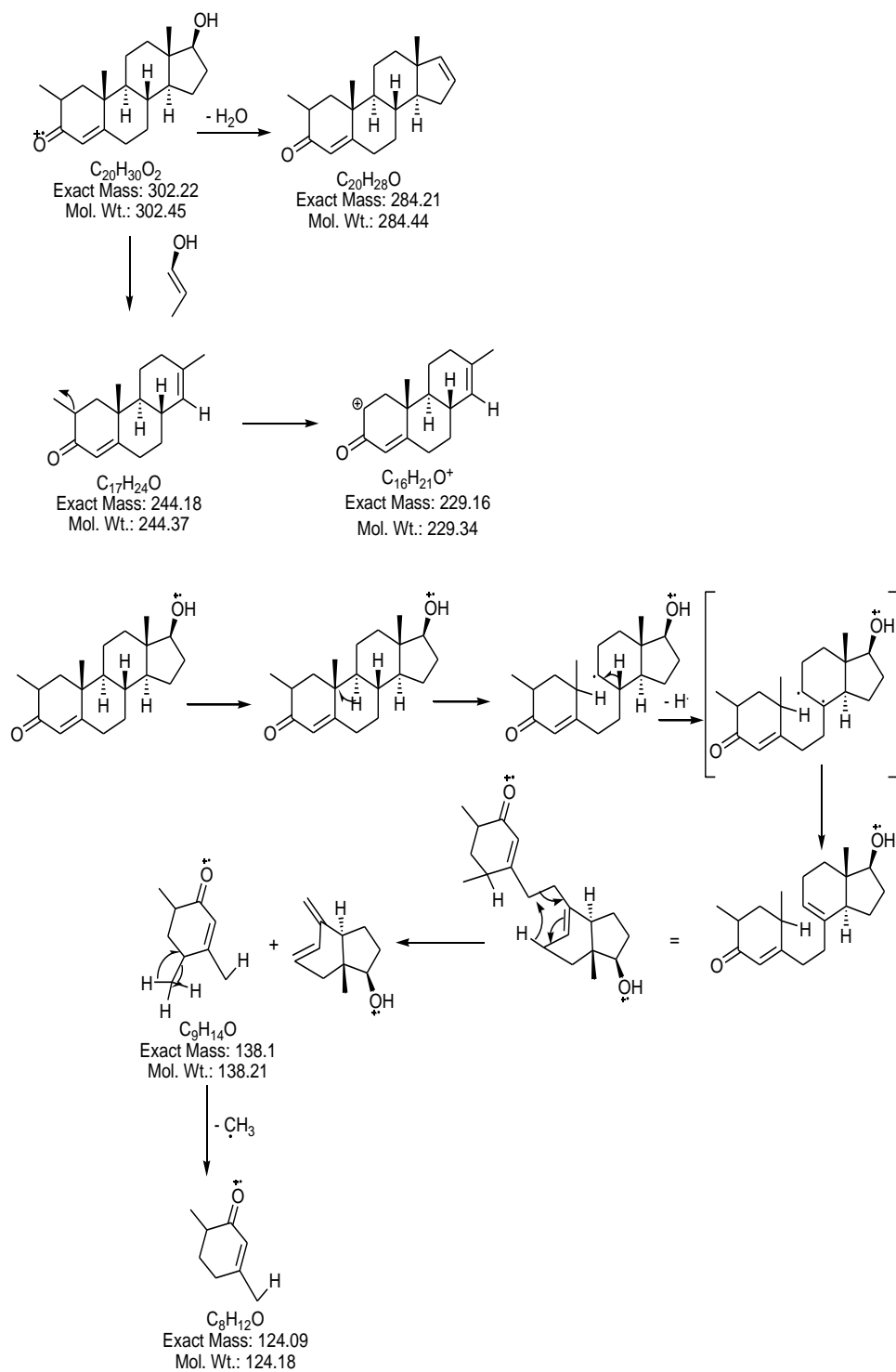
5. Gestrinone



6. THG



7. Methyltestosterone



Appendix A2

Plots of the peak areas obtained for the quantitation ion for each of the prohormones over the injection msss range of 50 to 500 pg

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- Figure A2. a)-c). Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5(10)-estrene-3b,17b-diol, 4-estrene-3b,17b-diol, 5(10)-estrene-3,17-dione, over the injection mass range of 50 to 500 pg.*
- Figure A2. d)-f). Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 4-androstene-3b,17b-diol, DHEA, 5(6)-androstene-3b,17b-diol, over the injection mass range of 50 to 500 pg.*
- Figure A2. g)-i). Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5a-androstane-3b,17b-diol, 5a-androstane-3,17-dione, nandrolone, over the injection mass range of 50 to 500 pg.*
- Figure A2. j)-l). Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5(6)-androstene-3,17-dione, testosterone, 1,4-androstane-3,17-dione, over the injection mass range of 50 to 500 pg.*

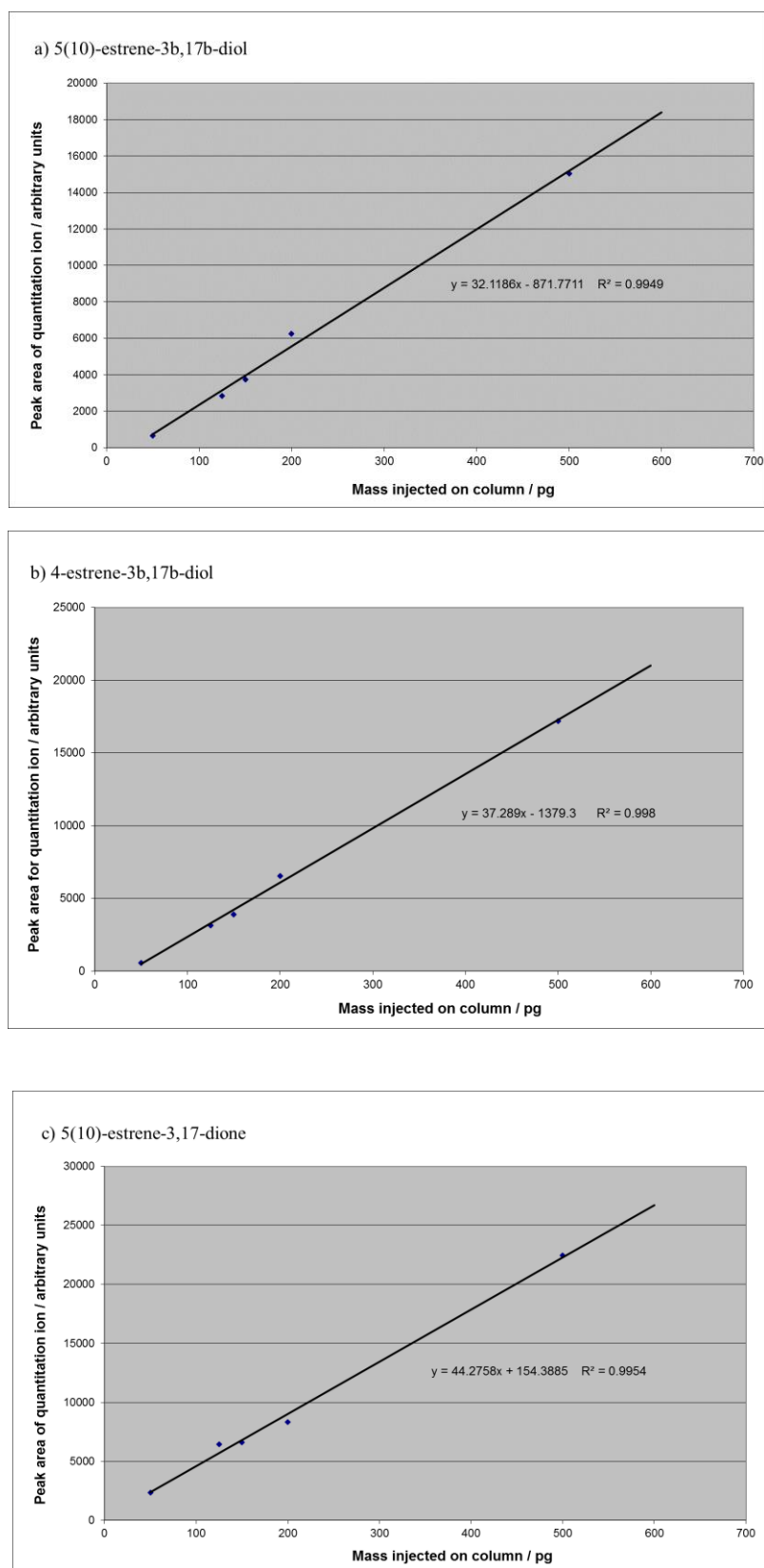


Figure A2. a)-c). Plots of the peak areas obtained for the quantitation ion for each of the prohormones over the injection mass range of 50 to 500 pg.

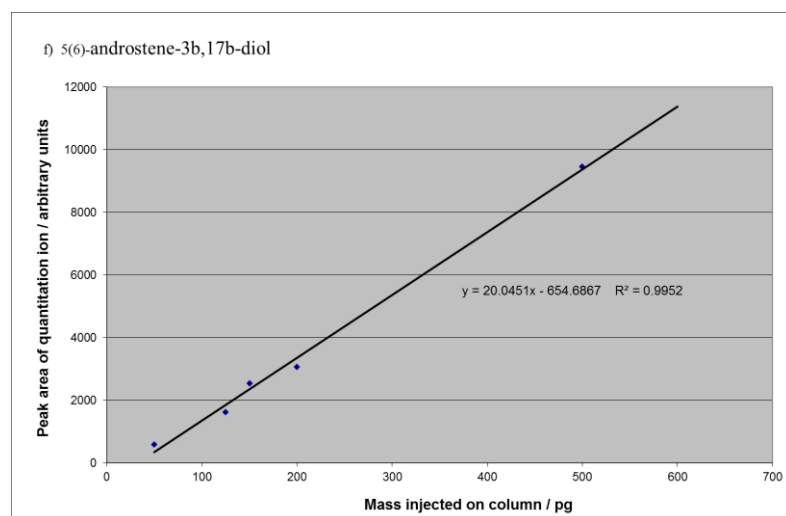
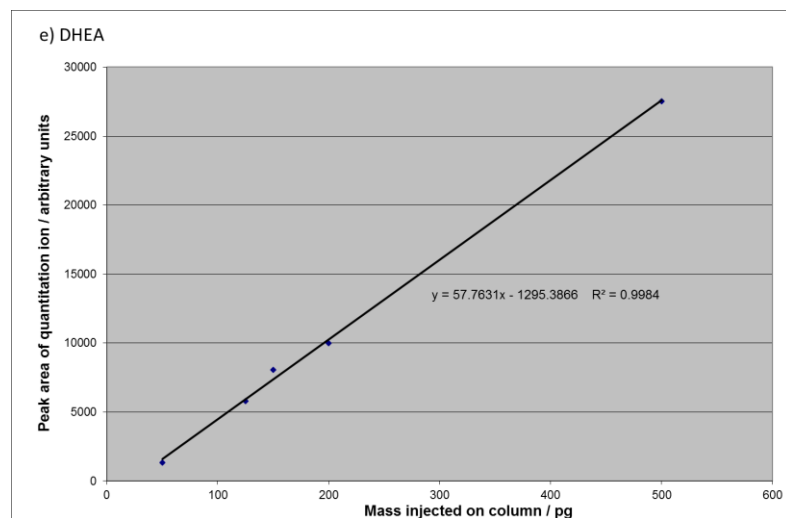
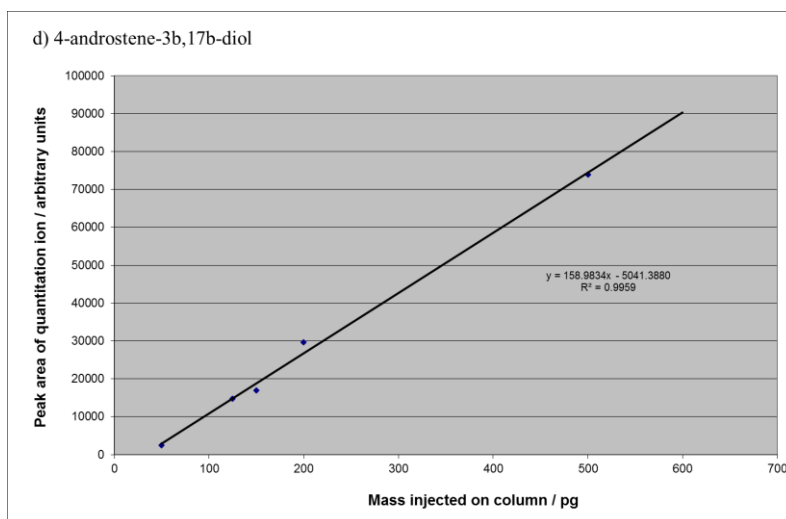


Figure A2. d)-f). Plots of the peak areas (arbitrary units) obtained for the quantitation ion for each of the prohormones, over the injection mass range of 50 to 500 pg.

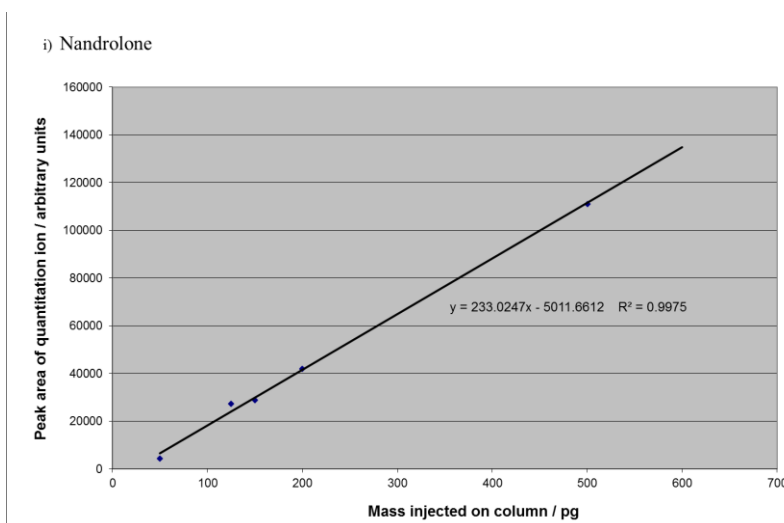
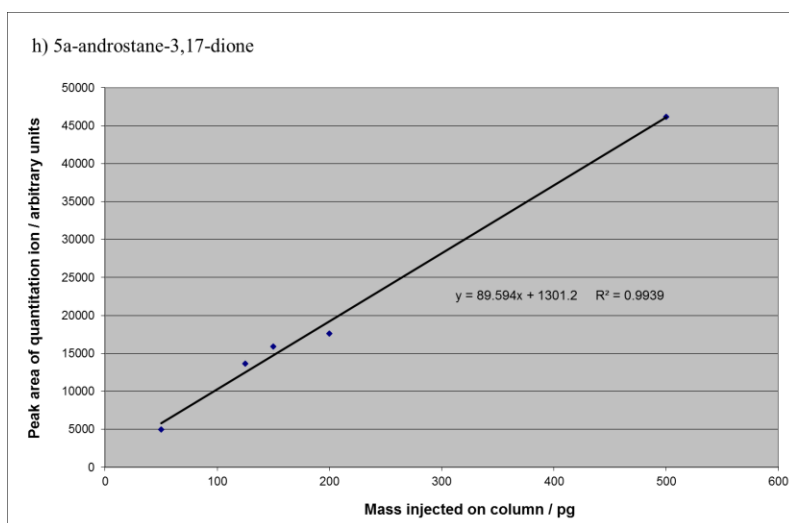
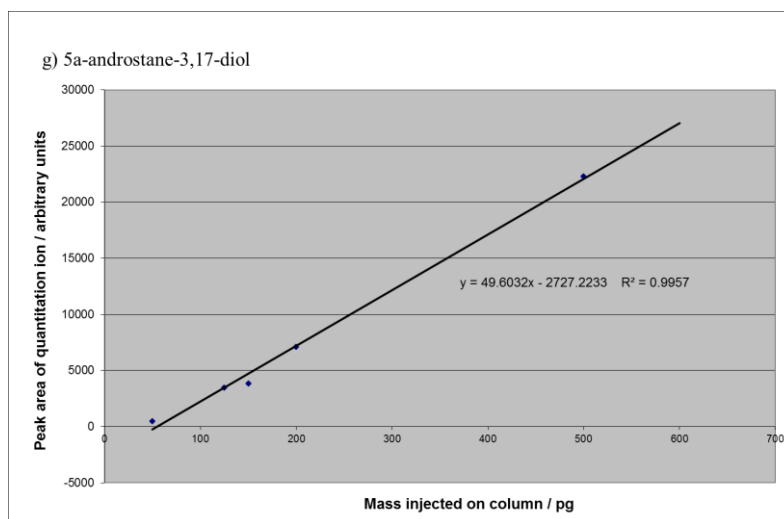


Figure A2. g)-i) Plots of the peak areas (arbitrary units) obtained for the quantitation ion for each of the prohormones, over the injection mass range of 50 to 500 pg.

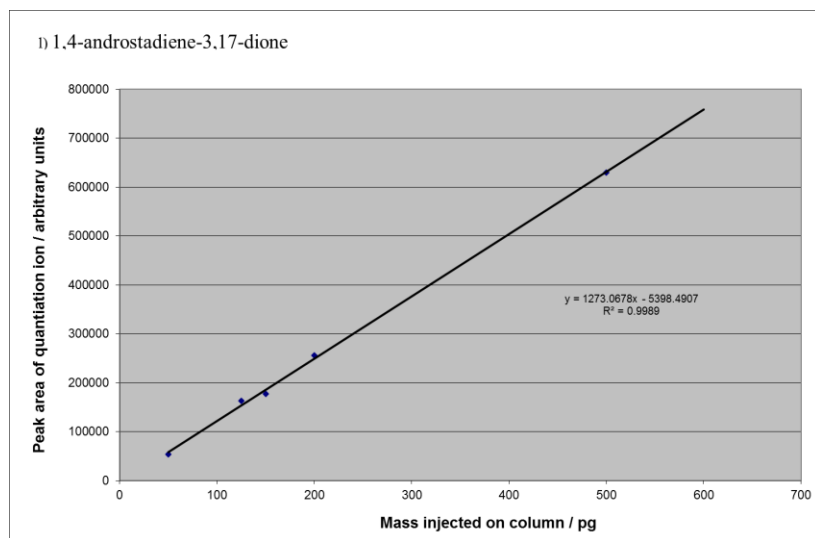
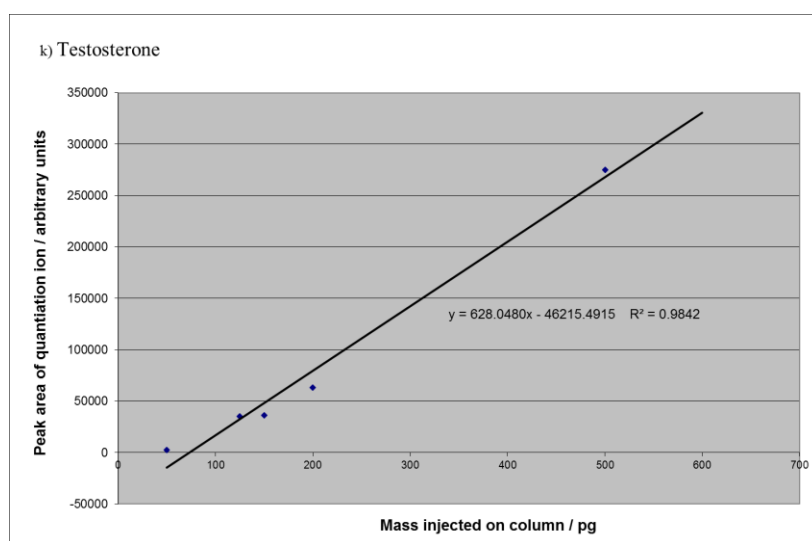
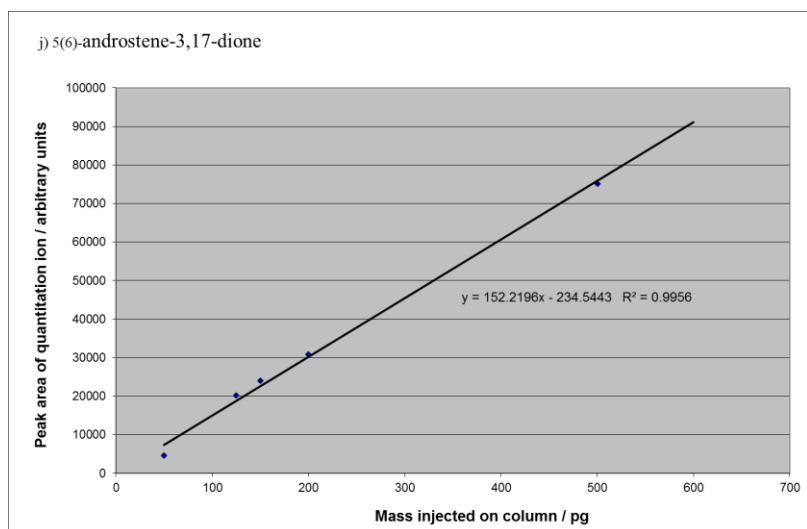


Figure A2. j)-l) Plots of the peak areas obtained for the quantitation ion for each of the prohormones, over the injection mass range of 50 to 500 pg.

Appendix A3

Plots of the peak areas obtained for the quantitation ion of each prohormones and designer steroids over the mass range of 25 to 250 pg

Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones and designer steroids versus the internal standard, over the mass range 25 to 100 pg

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Figure A3.1 a)-c) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5(10)-estrene-3b,17b-diol [1], 4-estrene-3b,17b-diol [2], 5(10)-estrene-3,17-dione [3], over the mass range 25 to 250 pg.

Figure A3.1 d)-f) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 4-androstene-3b,17b-diol [4], DHEA [5], 5(6)-androstene-3b,17b-diol [6], over the mass range of 25 to 250 pg.

Figure A3.1 g)-i) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5a-androstane-3b,17b-diol [7], 5a-androstane-3,17-dione [8], nandrolone [9] over the mass range of 25 to 250 pg.

Figure A3.1 j)-l) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5(6)-androstene-3,17-dione [10], testosterone [11], 1,4-androstane-3,17-dione [12] over the mass range of 25 to 250 pg.

Figure A3.2 a)-c) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 5(10)-estrene-3b,17b-diol [1], 4-estrene-3b,17b-diol [2], 5(10)-estrene-3,17-dione [3], versus the internal standard, over the mass range 25 to 100 pg.

Figure A3.2 d)-f) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 4-androstene-3b,17b-diol [4], DHEA [5], 5(6)-androstene-3b,17b-diol [6], versus the internal standard, over the mass range of 25 to 100 pg.

Figure A3.2 g)-i) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 5a-androstane-3b,17b-diol [7], 5a-androstane-3,17-dione [8], nandrolone [9], versus the internal standard, over the mass range of 25 to 100 pg.

Figure A3.2 j)-l) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 5(6)-androstene-3,17-dione [10],

testosterone [11], 1,4-androstane-3,17-dione [12], versus the internal standard, over the mass range of 25 to 100 pg.

Figure A3.3 a)-c) Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epitestosterone, Xtren and trenazone, over the mass range 25 to 500 pg.

Figure A3.3 a)-c) Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epistane, gestrinone and THG, over the mass range 25 to 500 pg.

Figure A3.4 a)-c) Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epitestosterone, Xtren and trenazone, over the mass range 25 to 500 pg (data point at 250 pg has been removed).

Figure A3.4 a)-c) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the designer steroids: epistane [16], gestrinone [17], THG [18], versus the internal standard, over the mass range of 25 to 100 pg.

Figure A3.4 d)-f) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the designer steroids : epitestosterone [13], Xtren [14], trenazone [15], versus the internal standard, over the mass range of 25 to 100 pg.

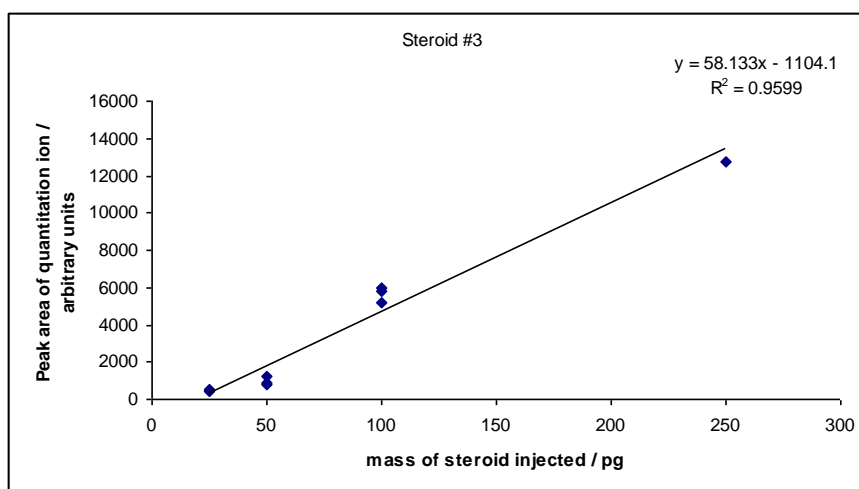
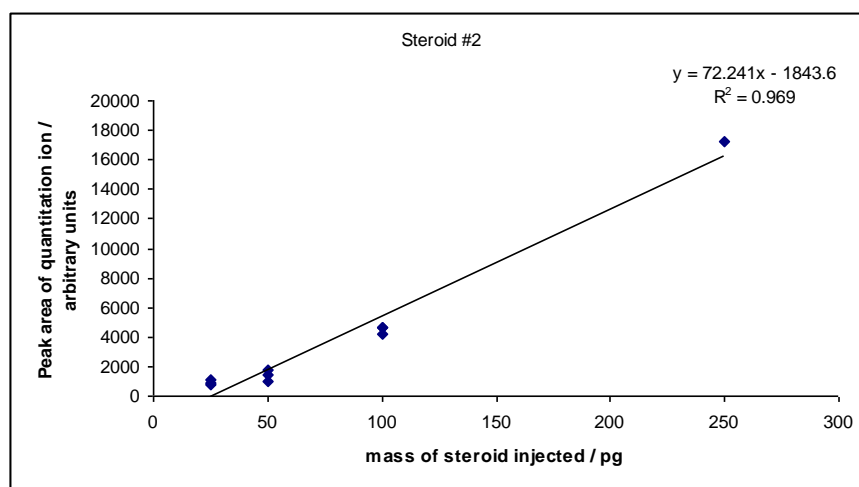
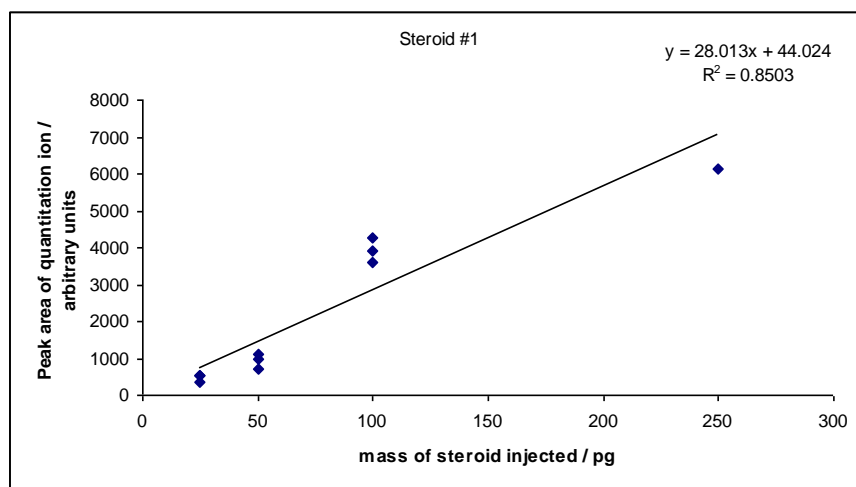


Figure A3.1 a)-c) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5(10)-estrene-3b,17b-diol [1], 4-estrene-3b,17b-diol [2], 5(10)-estrene-3,17-dione [3], over the mass range 25 to 250 pg.

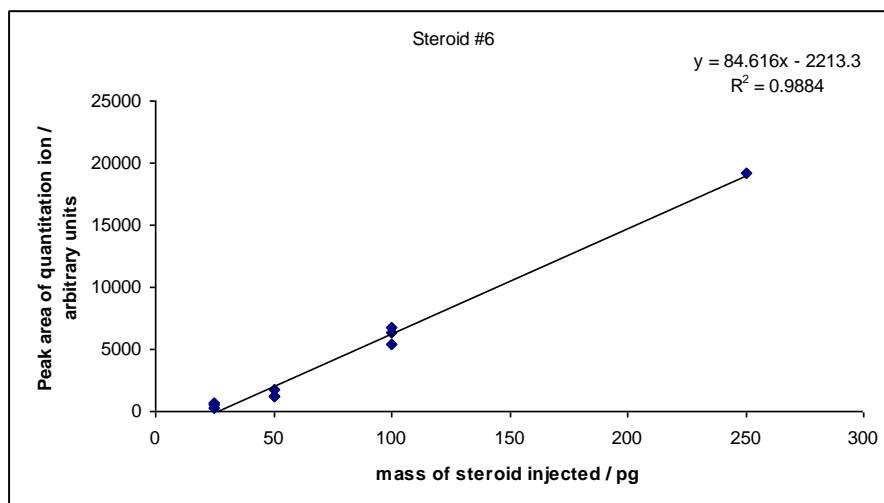
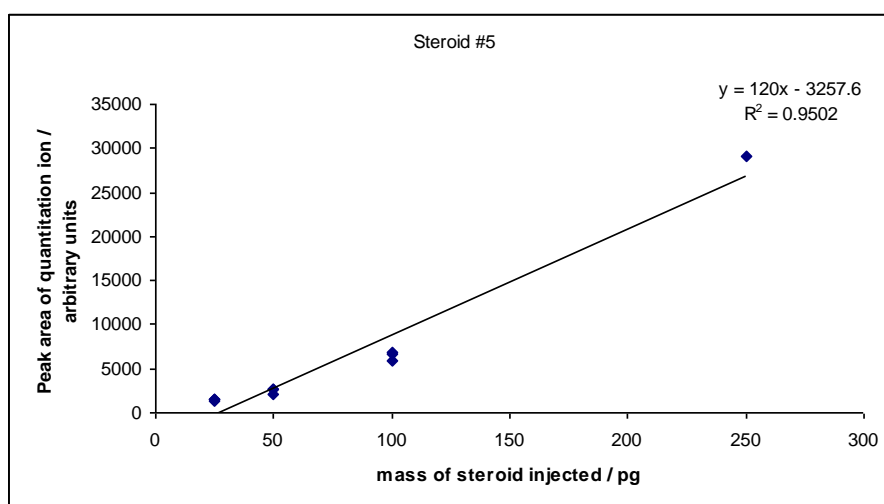
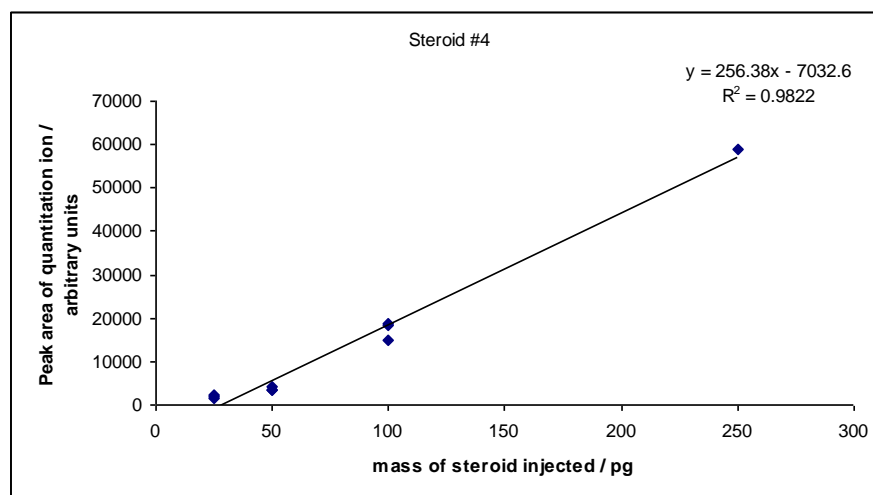


Figure A3.1 d)-f) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 4-androstene-3b,17b-diol [4], DHEA [5], 5(6)-androstene-3b,17b-diol [6], over the mass range of 25 to 250 pg.

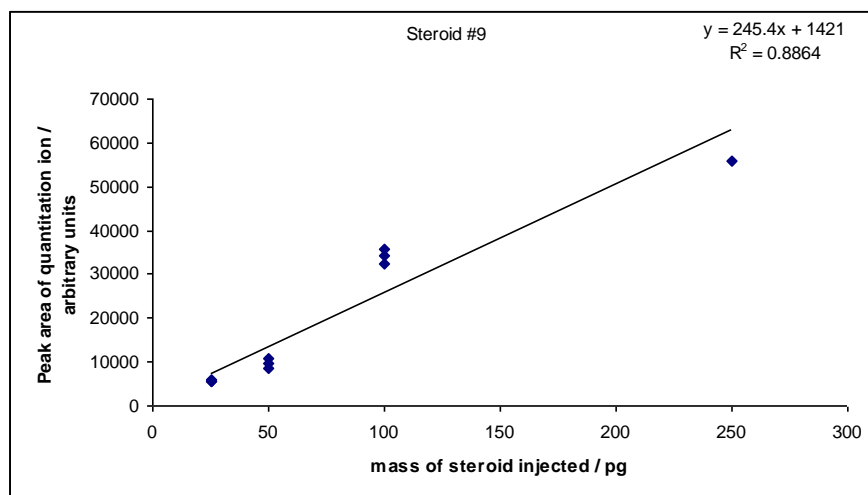
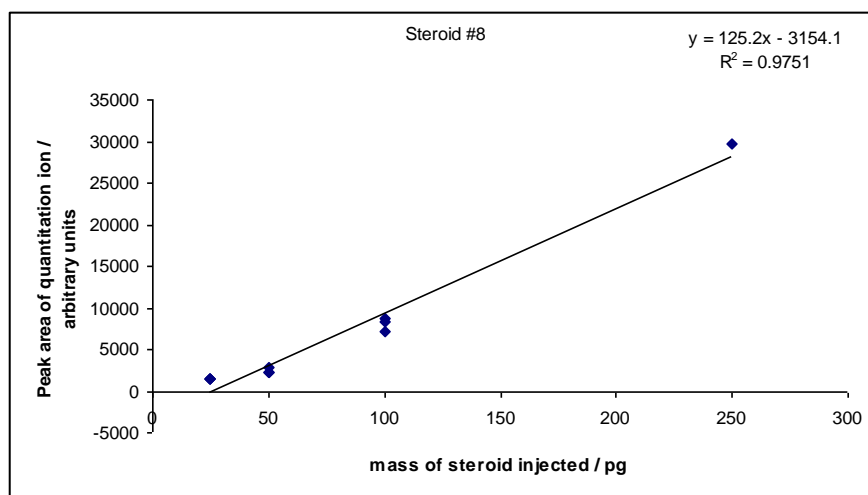
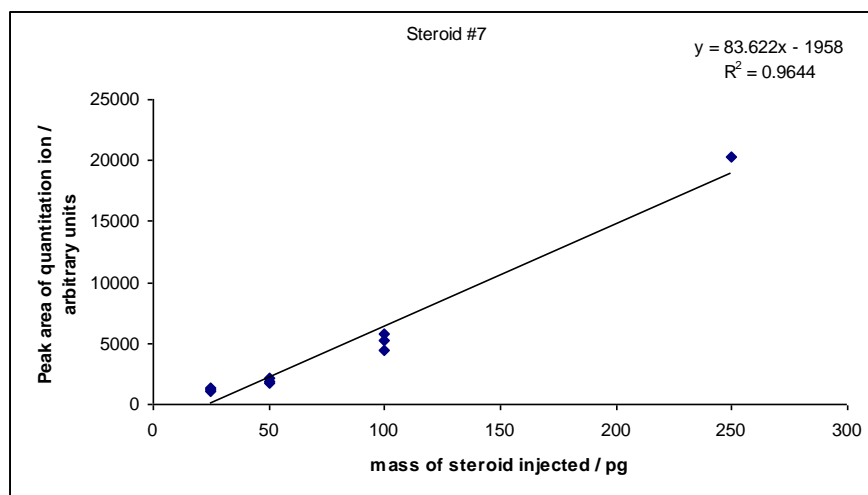


Figure A3.1 g)-i) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5a-androstane-3b,17b-diol [7], 5a-androstane-3,17-dione [8], nandrolone [9] over the mass range of 25 to 250 pg.

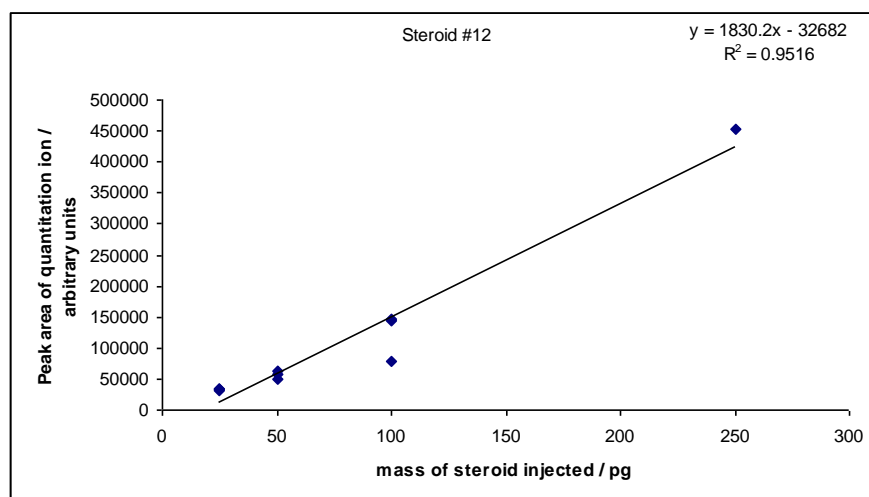
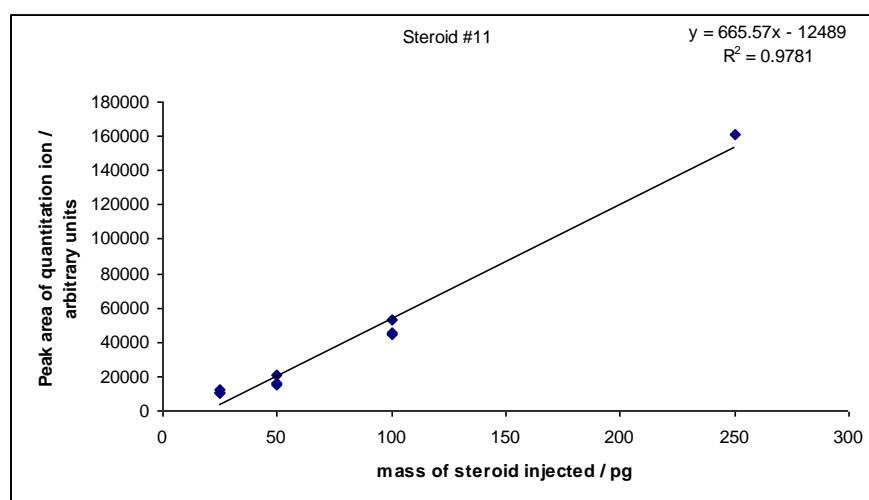
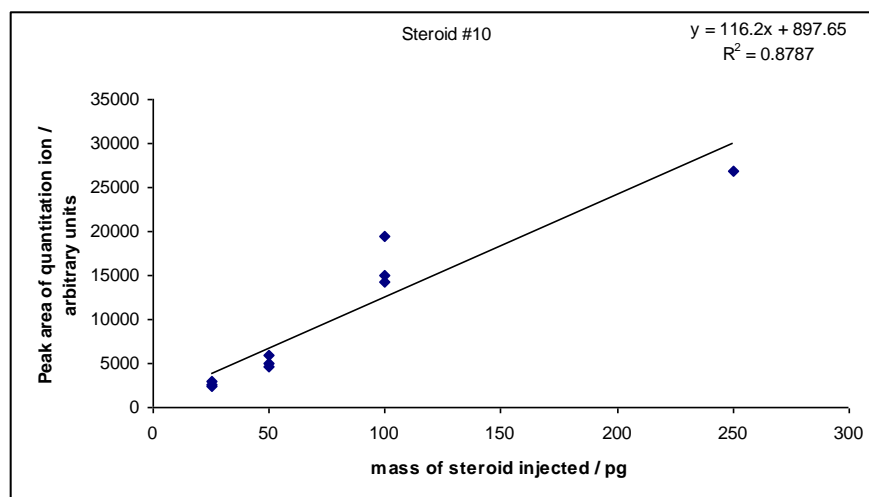


Figure A3.1 j)-l) Plots of the peak areas obtained for the quantitation ion for each of the prohormones: 5(6)-androstene-3,17-dione [10], testosterone [11], 1,4-androstane-3,17-dione [12] over the mass range of 25 to 250 pg.

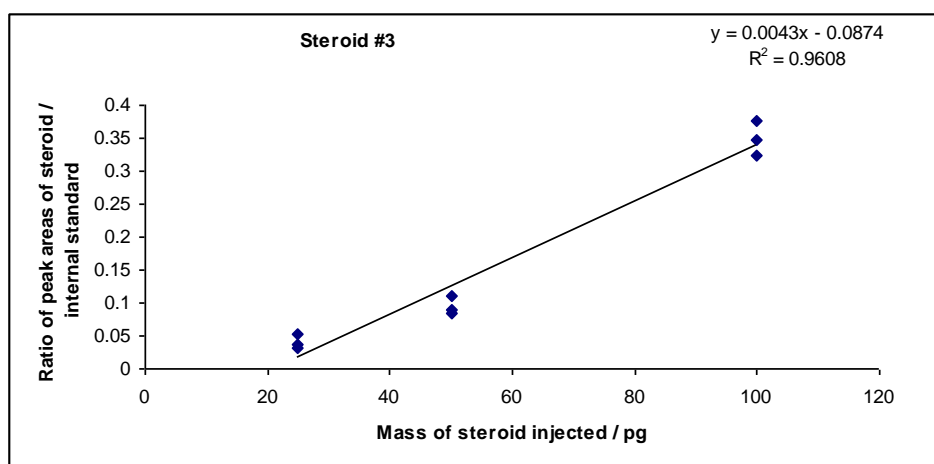
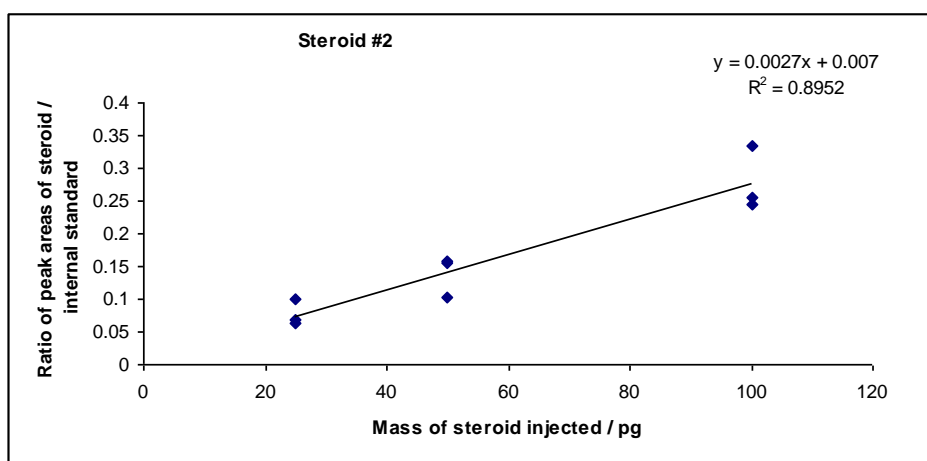
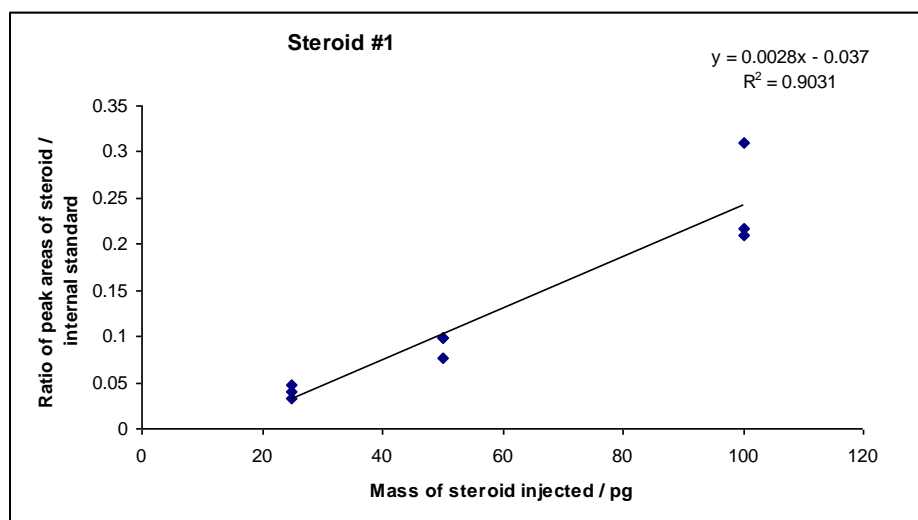


Figure A3.2. a)-c) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 5(10)-estrene-3b,17b-diol [1], 4-estrene-3b,17b-diol [2], 5(10)-estrene-3,17-dione [3], versus the internal standard, over the mass range 25 to 100 pg.

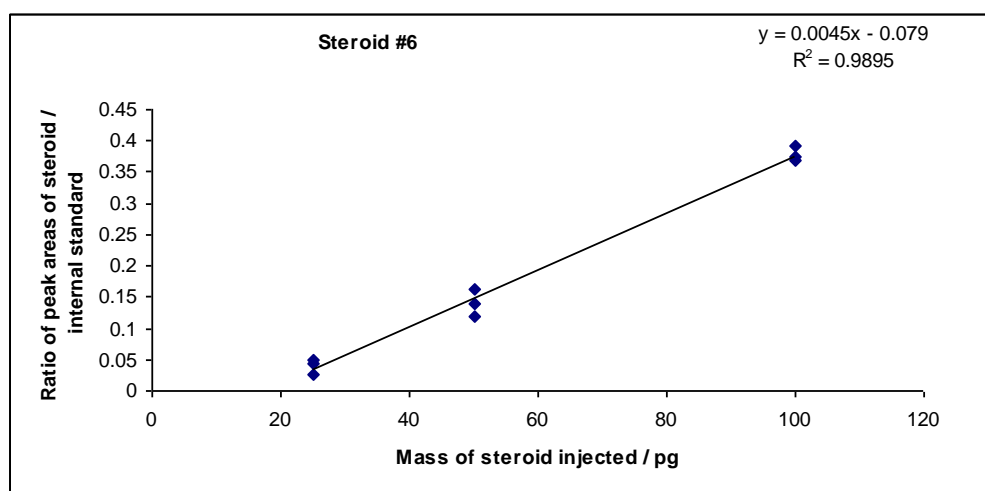
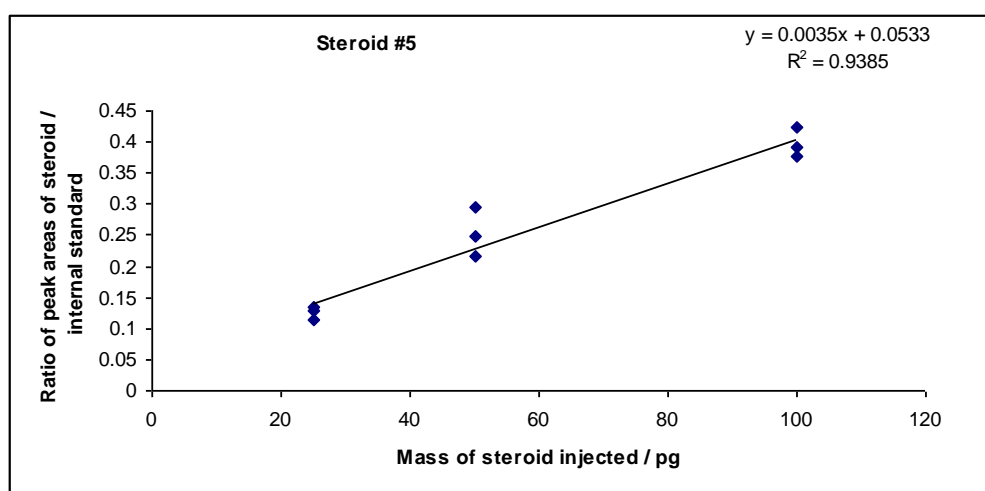
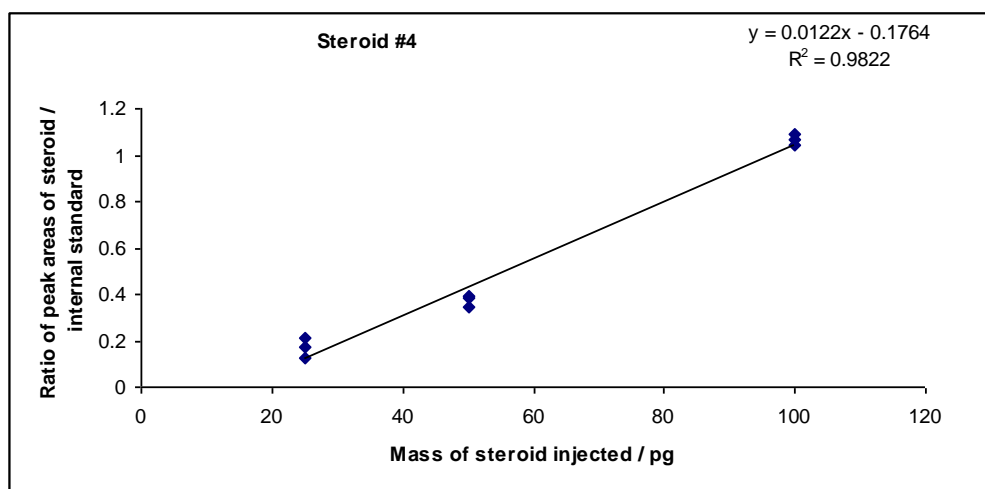


Figure A3.2 d)-f) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 4-androstene-3b,17b-diol [4], DHEA [5], 5(6)-androstene-3b,17b-diol [6], versus the internal standard, over the mass range of 25 to 100 pg.

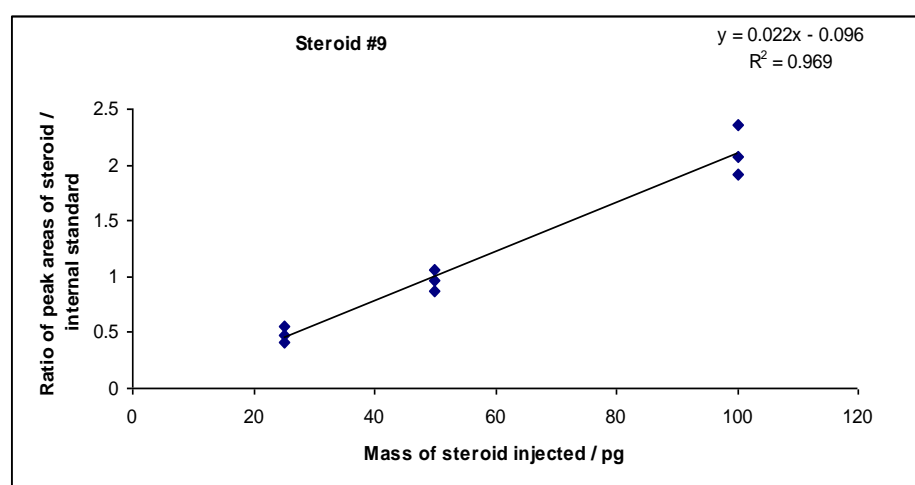
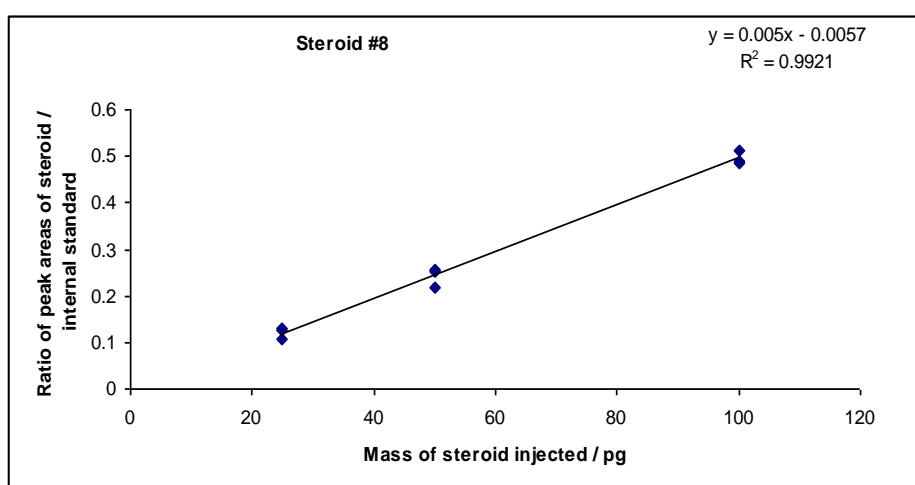
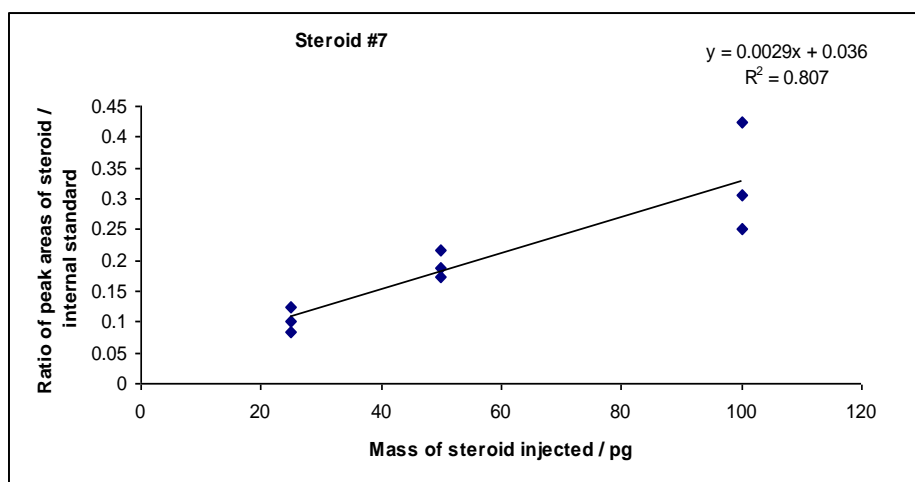


Figure A3.2 g)-i) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 5a-androstane-3b,17b-diol [7], 5a-androstane-3,17-dione [8], nandrolone [9], versus the internal standard, over the mass range of 25 to 100 pg.

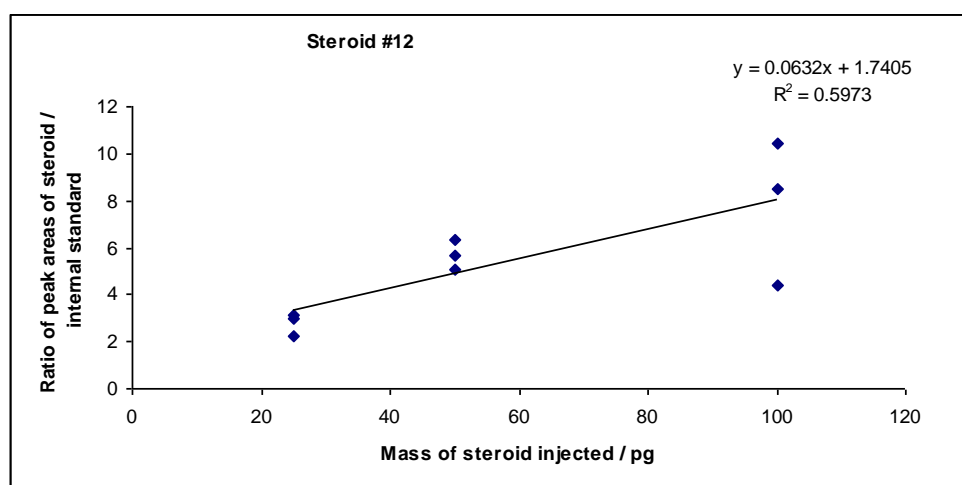
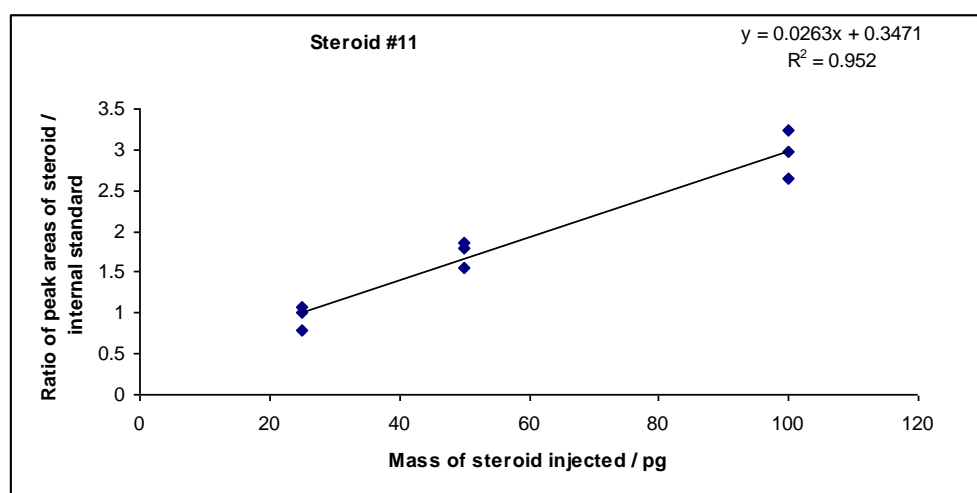
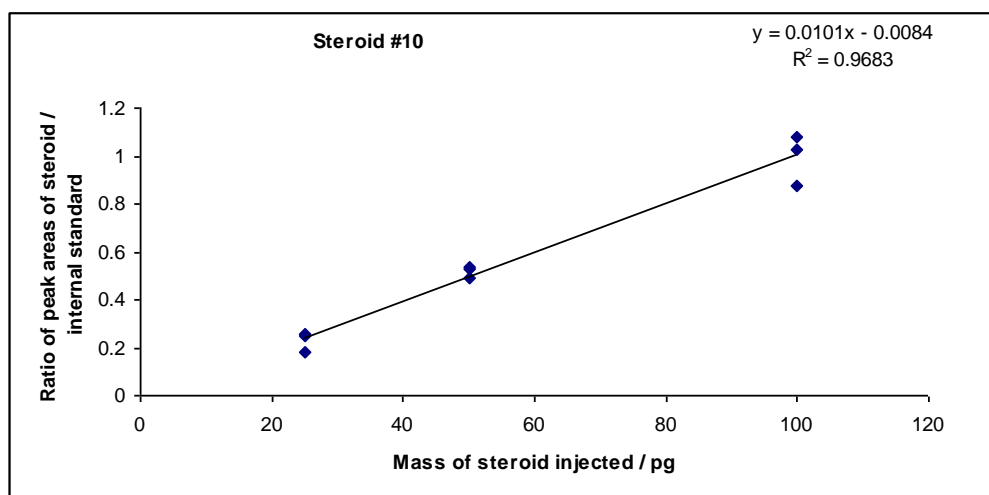


Figure A3.2 j)-l) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the prohormones: 5(6)-androstene-3,17-dione [10], testosterone [11], 1,4-androstane-3,17-dione [12], versus the internal standard, over the mass range of 25 to 100 pg.

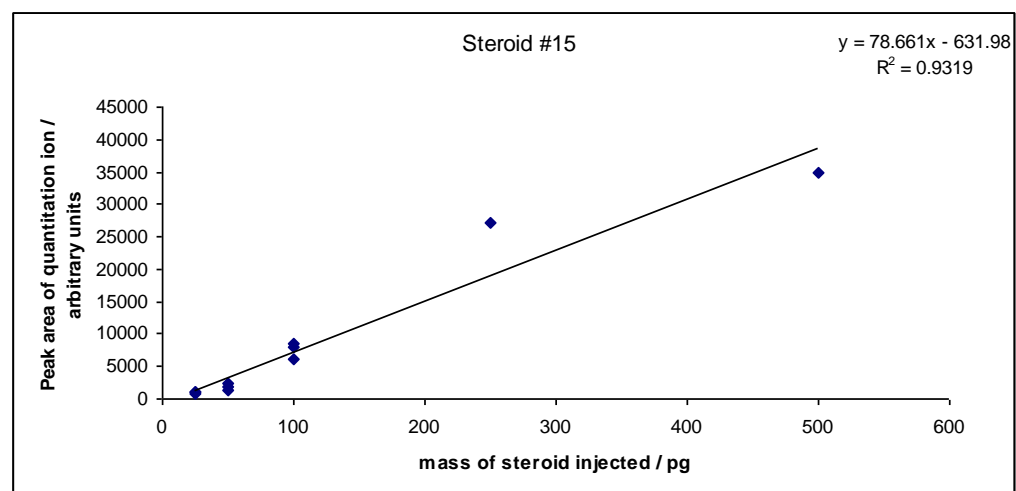
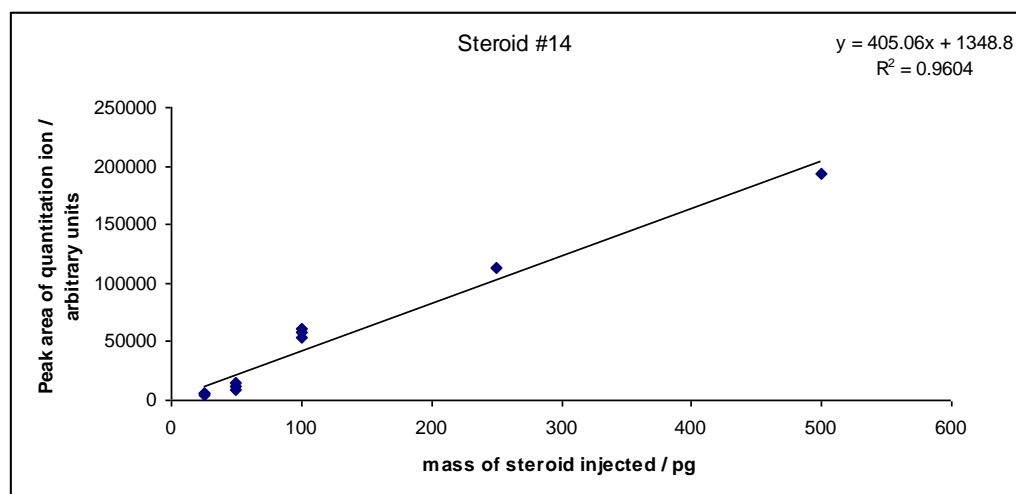
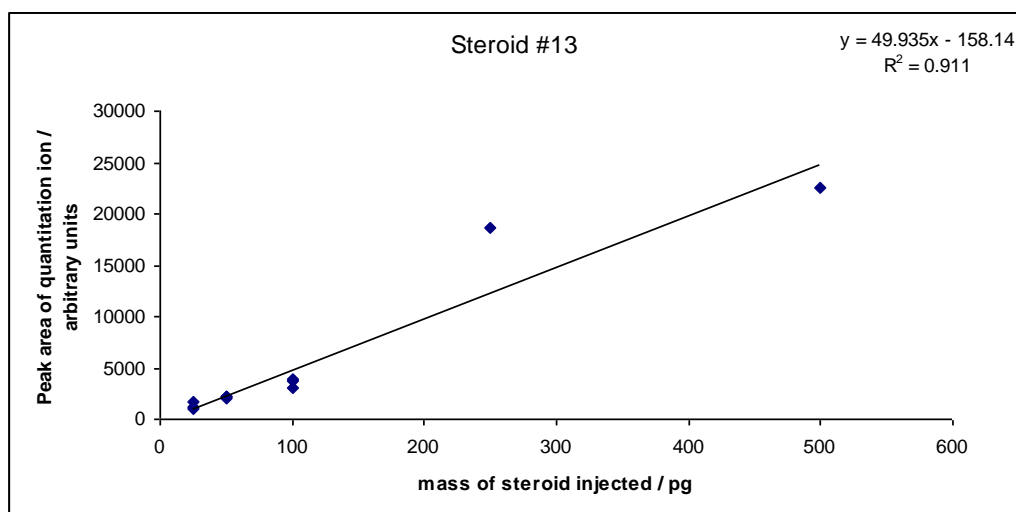


Figure A3.3 a)-c) Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epitestosterone [13], Xtren [14] and trenazone [15], over the mass range 25 to 500 pg.

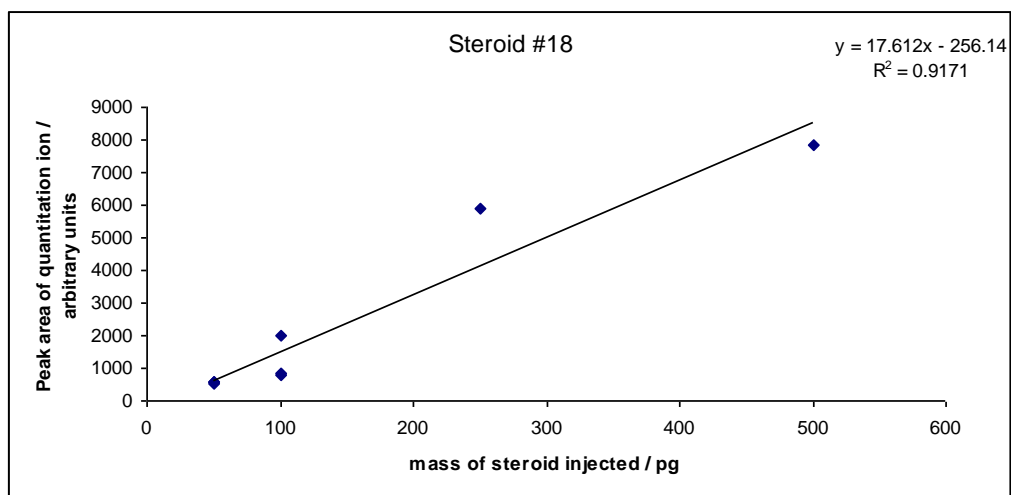
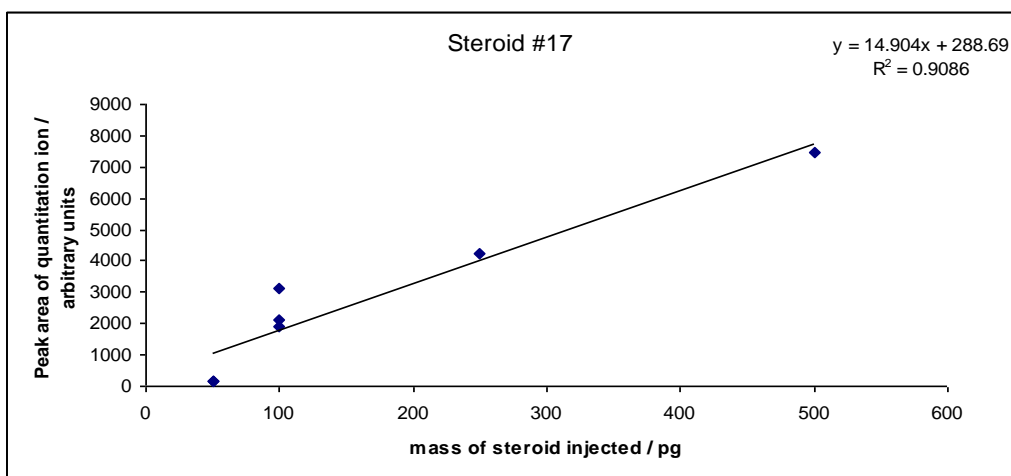
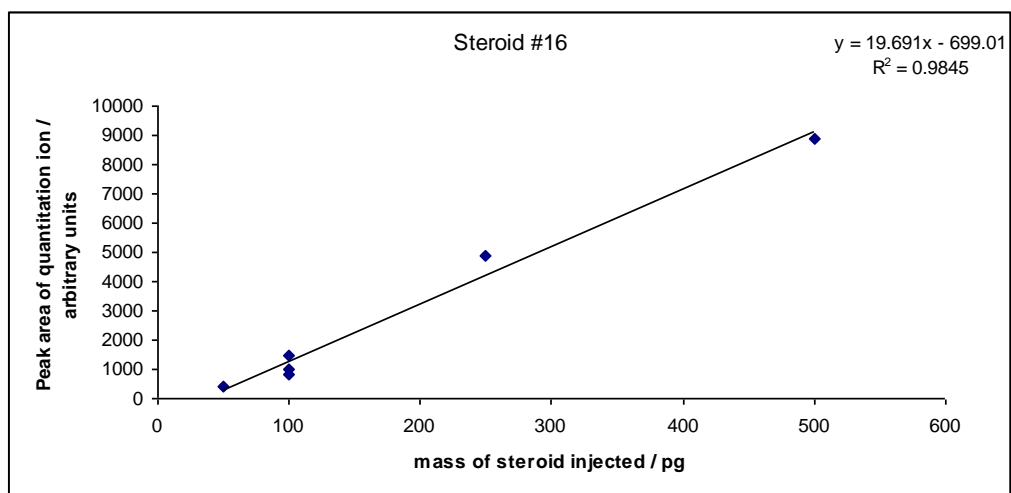


Figure A3.3 d)-f) Plots of the peak areas obtained for the quantitation ion for each of the designer steroids: epistane[16], gestrinone [17] & THG [18], over the mass range 25 to 500 pg.

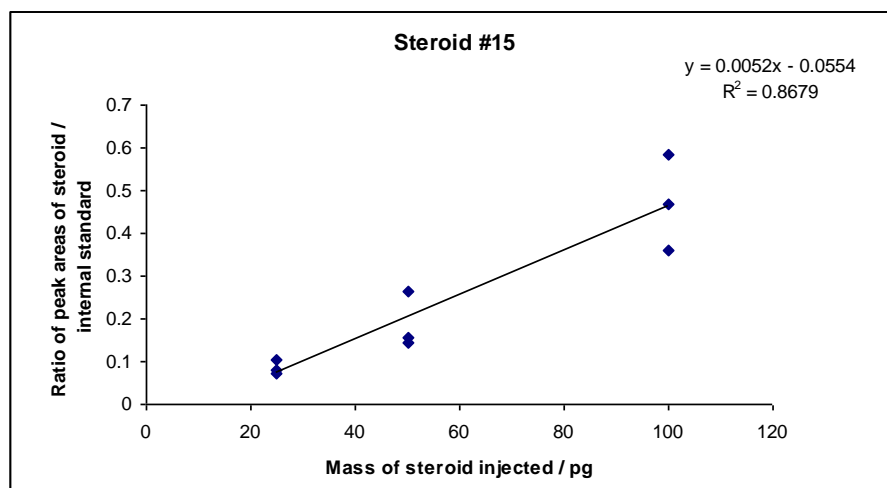
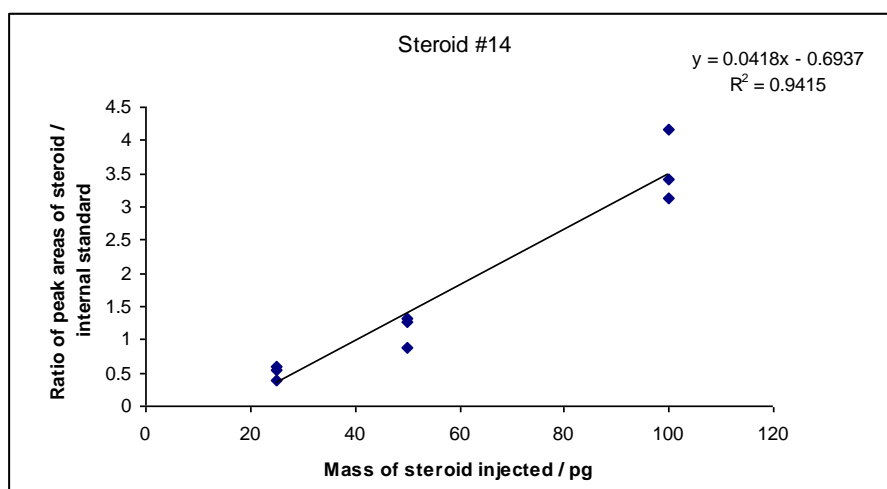
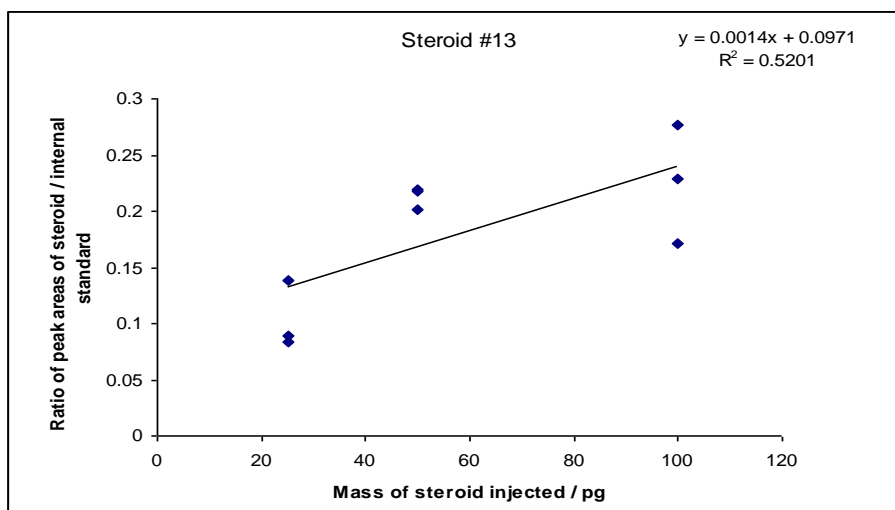


Figure A3.4 a)-c) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the designer steroids : epitestosterone [13], Xtren [14], trenazone [15], versus the internal standard, over the mass range of 25 to 100 pg.

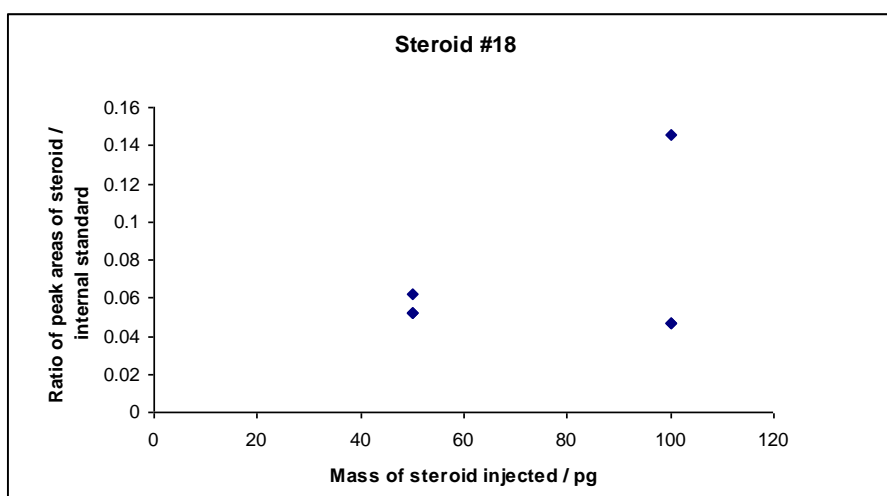
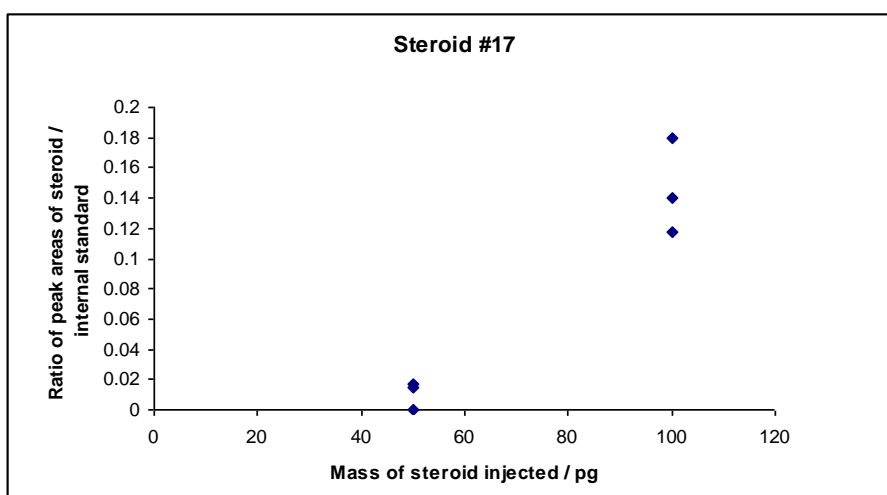
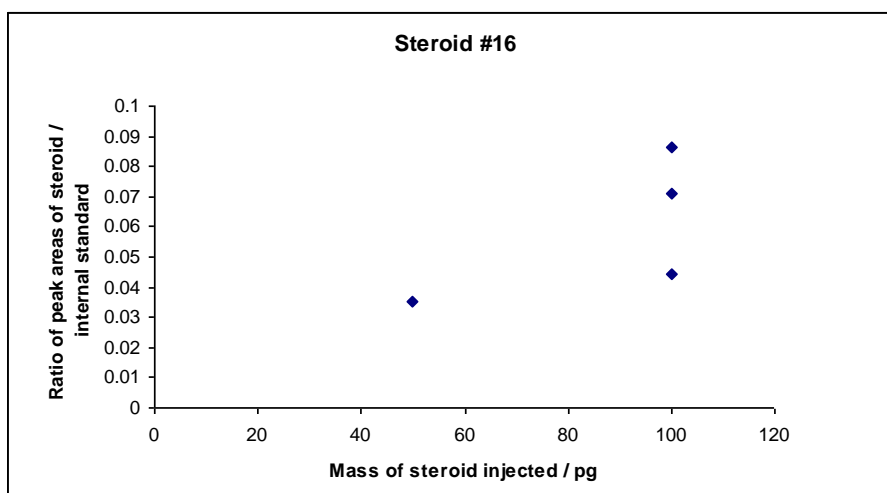


Figure A3.4 d)-f) Plots of the ratio of the peak areas obtained for the quantitation ion for each of the designer steroids: epistane [16], gestrinone [17], THG [18], versus the internal standard, over the mass range of 25 to 100 pg.

Appendix A4

Publication